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# ARCHIVES OF BIOCHEMISTRY

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# Isolation of a New Lipoprotein, Lipovitellenin, from Egg Yolk

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Received April 3, 1946

## INTRODUCTION

A new procedure for the preparation of lipovitellin from egg yolk has recently been reported (1). The procedure consists essentially of throwing out the lipovitellin by centrifuging egg yolk diluted with two volumes of water in a Sharples centrifuge, drying by lyophilization, extracting the dried precipitate with ether, dissolving the lipovitellin in 10% NaCl solution, and reprecipitating by removal of the salt by dialysis. The yield of lipovitellin by this method was considerably lower than the amounts indicated in the literature (2).

The aqueous emulsion from which the lipovitellin had been removed by centrifugation has been found to contain, in addition to the water-soluble proteins and the yolk lipids, a second lipoprotein which has been called lipovitellenin. Lipovitellin and lipovitellenin differ from each other in the ratio of combined protein and lipid, in solubility behavior, in general stability, and in the composition of the protein components, vitellin and vitellenin. Both are phosphoproteins, but they differ markedly in phosphorus contents.

## EXPERIMENTAL

### *Preparation and Properties of Lipovitellenin*

Lipovitellenin was separated from the other yolk constituents by the following procedure: Egg yolk emulsion prepared from the yolks of fresh eggs was diluted with two volumes of water and centrifuged in a Sharples centrifuge (bowl speed 40,000 r.p.m.) to remove the lipovitellin. The supernatant, containing livetin, fats and the substance in question, was extracted with two volumes of ether in separatory funnels. Three layers separated; an aqueous layer containing the livetin, an ether layer containing the lipids, and a third layer of insoluble material which separated between

\* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.



the other two. The aqueous and ether layers were removed and the solid layer extracted repeatedly with ether (2 to 3 volumes) until the ether extract was colorless. The precipitate at this time was a yellowish, somewhat gelatinous mass, which, still containing residual ether, dissolved in two volumes of 10% NaCl solution to a clear golden yellow solution.

The dissolved material was precipitated when the salt was removed by dialysis. The precipitate now dissolved to a stable opalescent solution in 10% salt. However, if the solution was saturated with ether, the clear yellow solution mentioned above again resulted. If the solution was allowed to stand exposed to air so that the ether gradually evaporated, the opalescence again appeared and could again be cleared up by addition of ether.

The precipitate obtained by dialysis of the ether-saturated salt solution was emulsified in water and dried from the frozen state (preparations 1, 2 and 3, Table I). Preparations 4, 5, 6 and 7 (Table I) were prepared as described, with the added

TABLE I  
*Composition of Lipovitellenin and Vitellenin\**

Prep. No.	Alcohol-Extractable Phospholipid	Nitrogen in (4)			Phosphorus in (5)			Sulfur in (6)	
		Lipovitellenin	Vitellenin	Phospholipid	Lipovitellenin	Vitellenin	Phospholipid	Lipovitellenin	Vitellenin
	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
1	41.5	9.98	15.4	1.80	1.74	0.29	3.76		
2	41.0	10.00	15.4	1.65	1.71	0.29	3.51		
3	40.0	9.90	15.6	1.76		0.31	3.62		
4	38.6	10.03	15.7	-	1.71	0.29		0.61	0.92
5	37.3	—	15.5	1.82	1.68	0.24	3.71	0.60	0.86
6	38.0	9.90	15.6	1.79	1.74	0.30	3.77	0.59	0.91
7	39.0	—	15.4	1.75	—	0.31	3.69	0.62	0.94
8	38.5	9.90	15.6	-	1.70	0.29			0.90
9	32.7	10.96	15.7	1.83	1.56	0.70	3.72		
10	37.0	10.0	15.5		1.69	0.28		0.59	0.92
11	37.4	10.1	15.4		1.71	0.30		0.61	0.92
12	37.0	9.9	15.6		1.70	0.27			
13	38.4	10.2	15.3		1.71	0.26			
14†	16.7	13.0	15.6		1.46	1.10			1.00

\* All values on dry basis.

† Preparation 14 includes, for comparison, values for lipovitellin and vitellin (1, 3).

precaution that the dried material was again extracted with ether before weighing and before analysis. Sample 8 was prepared by drying the precipitate, which results on extraction of the Sharples supernatant, by repeated extraction with ether until fairly dry and then drying further over phosphorus pentoxide *in vacuo*. All the preparations showed the same specific solubility characteristic of emulsifying in 10% NaCl but dissolving to a clear solution when the solution was saturated with ether. Occa-

sionally a scum of insoluble material which comprised an insignificant amount of the total (<1%) was present and collected on the surface of the salt solution or between the ether and salt solutions.

The dried material prepared as described was extracted repeatedly (6 or 7 times) with cold ethyl alcohol (40 cc./g. of sample). The alcohol-soluble material was dried and weighed. It comprised from 36 to 41% of the weight of the original sample (Table I). After extraction the alcohol-soluble material was also soluble in ether while the residue was insoluble in all neutral solvents tested. These properties identify the original material as a lipoprotein with the same general properties as lipovitellin but containing different proportions of lipid and protein. As already stated, this lipoprotein has been named lipovitellenin.

Extraction of the lipoprotein with acetone also resulted in decomposition. Only 8–10% of the lipid was extracted by the acetone, probably because of the phospholipid nature of the fatty constituent. After acetone extraction, however, 15–20% of the weight of the remaining material was soluble in ether, presumably as a result of the decomposing effect of the acetone. The material was also insoluble in NaCl solution after acetone extraction. This behavior is in contrast to lipovitellin, which is stable to acetone as well as to ether.

The lipovitellenin is also unstable in the dry form. As already stated, after drying by lyophilization the solubility characteristics were retained. However, after standing at room temperature in a closed container for a few days, the material can no longer be dispersed to a stable colloidal solution in salt solution nor to a clear solution in ether-saturated salt solution. The material now swells to a yellow transparent mass in 10% salt solution and can readily be centrifuged from the remaining solvent. In ether-saturated salt solution the swelling is more pronounced and, on standing, a firm rubbery mass results. This loss of solubility on exposure to air in dry form appears to be an "all or none" reaction. The change apparently takes place rapidly and the loss of solubility has never been observed to be a partial one.

Lipovitellenin can be stored at room temperature in 10% salt solution saturated with ether without decomposition. The solubility characteristics do not change and analysis shows no change after one month of storage (preparation 10, Table I).

Lipovitellenin readily dissolves in alkali. A solution was prepared by suspending 4 g. of lipovitellenin (from preparation 10) in 200 cc. of distilled water and adding *N* KOH dropwise to pH 9.0. The solution was clear and not obviously viscous. On adding *N* sulfuric acid dropwise to pH 5.5–6.0, the lipoprotein again precipitated. After washing

with water the precipitate was dried and weighed; 3.4 g. were recovered (preparation 11). It was apparently unchanged, as indicated by the unchanged solubility characteristics. The analytical figures likewise had not changed. Preparation 12 was prepared in a similar manner.

Preparation 9 (Table I) was prepared from egg yolk which had been frozen and thawed before the lipovitellenin was isolated. The yolk exhibited the typical rubbery consistency of such material. The preparation obtained as lipovitellenin was different from samples prepared from unfrozen yolk, in that it did not dissolve in ether-saturated salt solution to a clear solution but to a translucent milky suspension. The analytical values show that the amount of combined lipid was lower for this preparation, as was also indicated by a slightly higher nitrogen and a definitely lower phosphorus content. On analysis of the lipid-free protein a phosphorus value of 0.7% was obtained, which is higher than those for other preparations of vitellenin but lower than that for vitellin. It appears that this preparation may be a mixture of vitellin and vitellenin and that, after the yolks are frozen and thawed, the two lipoproteins are altered and cannot be separated in the usual manner.

#### *Preparation and Properties of Vitellenin*

Vitellenin, the protein component of lipovitellenin, was prepared by extracting the lipoprotein with alcohol, as already described, thus removing the lipid part of the complex. The solubility properties of the remaining protein, vitellenin, were found to be very similar to those of vitellin, the protein component of lipovitellin. It is a phosphoprotein which is insoluble in neutral solvents but dissolves in alkali at pH 9.0–10.0 to viscous solutions in a manner similar to casein or vitellin. At pH 11.0, or above, the viscosity decreases markedly. On acidification, vitellenin was again precipitated unchanged.

Table I presents phosphorus, nitrogen and sulfur values for a number of preparations. In the case of preparation No. 14, corresponding values for lipovitellin and vitellin are given. The significant differences in lipid content of the two lipoproteins and the marked difference in phosphorus content are apparent.

#### *Properties of the Lipid Component of Lipovitellenin*

The alcohol-extractable lipids combined in lipovitellenin are phospholipid in nature. Evaporation of the alcohol extract resulted in a

colorless waxy solid which was readily soluble in cold alcohol or ether and was precipitated from alcohol or ether solutions with acetone. The analytical values for nitrogen and phosphorus (Table I) approach the theoretical for lecithin but 0.3% of amino nitrogen was present, which represents one-sixth of the nitrogen contained in the material. It is, therefore, not pure lecithin.

### DISCUSSION

The lipoprotein described is obviously a different compound from lipovitellin, the lipoprotein previously isolated from egg yolk. The proportions of protein and phospholipid in the two lipoproteins are widely different, due to the presence of more than twice as much lipid in lipovitellenin. The lower nitrogen and the higher phosphorus contents also reflect the lower protein and the higher phospholipid contents of the lipovitellenin.

Lipovitellenin is not different from lipovitellin only in the ratio of lipid and protein. The analytical figures for vitellenin show that it contains less than one-third the amount of phosphorus contained in vitellin (1, 3). The sulfur content of vitellenin is 0.9%, which is somewhat lower than the 1.0% given by Chargaff (3) for vitellin. The protein component (vitellenin) of lipovitellenin, is, as shown primarily by the phosphorus content, different from that of vitellin, the protein of lipovitellin.

The present method of separation of the two lipoproteins, without denaturation of either, depends on the fact that, in the presence of the yolk fats, the lipovitellenin cannot be thrown down in a Sharples centrifuge after dilution of the egg yolk with two volumes of water, whereas lipovitellin is readily deposited as a precipitate. This difference in behavior appears to be due to the greater fat content of lipovitellenin, which increases its dispersibility in fatty emulsions to such an extent that it does not precipitate on centrifugation. This behavior is demonstrated by the fact that when the fats are removed from the emulsion with ether, the protein immediately precipitates. The peculiar solubility characteristic, of merely dispersing to a stable milky suspension in 10% salt but dissolving to a clear solution in ether-saturated salt solution, further demonstrates the influence of the large amount of fatty component on the dispersibility and solubility of the lipovitellenin.

Over half of the total phospholipids of egg yolk are bound to protein. Approximately 31% of the solids of egg yolk consist of lipoprotein; of this 31%, 17-18% contains 16% phospholipids, and the remaining 12-13% contains 38% phospholipid. The bound phospholipid in the two lipoproteins, therefore, amounts to 8% of the egg yolk solids. Separation of the crude uncombined phospholipid by cold acetone precipitation from the ether-soluble fats gave 5-6% of acetone-insoluble phospholipid.

Another fact that has become apparent in this work is the increased stability of the phospholipid when in combination with the protein. The combination appears to be a somewhat loose one, as judged by the ease with which it is broken. However, the typical browning of uncombined phospholipids on exposure to air takes place only very slowly in the phospholipid combined in the lipoproteins.

The use of the Sharples centrifuge has resulted in a simple, efficient method of separating egg yolk proteins. Lipovitellin and lipovitellenin are separated from one another (1), and the water-soluble protein(s) livetin is obtained in the aqueous part of the egg yolk, free of the two lipoproteins, and may then readily be isolated and fractionated by salt precipitation methods.

Some indications have been obtained that lipovitellenin may be concerned in the peculiar behavior of egg yolk in becoming "rubbery" after freezing and thawing. This process makes lipovitellenin less soluble, and experiments are in progress which are designed to determine the part played by lipovitellenin in this reaction.

The authors are indebted to L. M. White, Geraldine Secor, C. M. Johnson, E. F. Potter, Bernice Morrison and J. S. Furlow of this Laboratory for the nitrogen, moisture, phosphorus, sulfur and amino nitrogen determinations.

### SUMMARY

1. A new lipoprotein of egg yolk has been isolated.
2. The lipoprotein, which has been called lipovitellenin, contains 36-41% alcohol-extractable phospholipid as compared to 16-18% for the previously recognized lipoprotein, lipovitellin.
3. Lipovitellenin dissolves to an opalescent solution in 10% NaCl but to a clear yellow solution in 10% NaCl saturated with ethyl ether.
4. The new lipoprotein comprises roughly 40% of the total lipoprotein of egg yolk and 12-13% of the egg yolk solids.

5. The protein component (vitellenin) of the lipoprotein is a phosphoprotein similar to vitellin in general solubility behavior. It contains, however, less than one-third as much phosphorus as does vitellin.

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# Identification of the Bacteria-Inhibiting, Iron-Binding Protein of Egg White as Conalbumin

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## INTRODUCTION

Schade and Caroline (1) found that raw egg white contains a factor which inhibits the growth of *Shigella dysenteriae* and certain other microorganisms by fixing the iron in a form nutritionally unavailable to the bacteria. Dialyzed egg white retained its iron-binding activity, indicating the protein nature of the substance. In connection with our work dealing with the antibiotic, lysozyme (2), we have purified the factor responsible for this antibiotic activity and identified it as conalbumin.<sup>1</sup>

## EXPERIMENTAL

### *Method of Assay*

Schade and Caroline developed a microbiological method for the quantitative estimation of the antibacterial protein (1). They also found that the addition of ferrous iron to egg white or to concentrates of the substance in question gave rise to a pinkish tan coloration which was correlated with binding of iron and with antibiotic activity (1). We have used the colorimetric procedure for determining the concentration of the active substance. The essential facts have been substantiated, however, by microbiological analysis of the preparations by Schade and coworkers. The results of the two methods have in all cases verified each other.

The colorimetric analysis, as carried out in our laboratory, consisted of comparing the intensity of the color produced in various fractions of the starting material contained in borate-phosphate buffer, pH 8.9, on the addition of excess ferrous iron (200–250  $\gamma$  per 10 cc. of 0.2% protein solution). The transmission was measured at 450 m $\mu$  in a Coleman spectrophotometer.

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<sup>1</sup> This work was initiated at the suggestion of Schade and was carried out in cooperation with Schade and coworkers.



### Purification Procedures

It was found that crystalline egg albumin and crystalline lysozyme were inactive. The globulins of egg white, removed by adsorption on 4% bentonite, contained only traces of the iron-binding protein, as did also the globulins precipitated at 2 *M* ammonium sulfate concentration. The albumin fractions from the above separations retained their color-producing property with iron and, likewise, their antibiotic activity. Further ammonium sulfate fractionation of the albumin fraction showed that essentially all of the active substance was precipitated between 2.3 and 2.6 *M*, together with most of the egg white albumins.

Since the possible identity of the iron-binding protein with any of the known proteins of egg white, with the exception of conalbumin and ovomucoid, had been eliminated, we undertook first the preparation of conalbumin. Longsworth *et al.* (3) stated that "when the albumin fraction is acidified to a pH below 4, a precipitate forms more or less slowly," which, on electrophoretic examination, was found to be conalbumin.<sup>2</sup> We found, however, that acidification of a 5% salt-free solution of the albumin fraction originally precipitated from egg white at pH 5.8 by 2.3–2.6 *M* ammonium sulfate (concentration) to pH 3 with sulfuric acid failed to cause any protein precipitation during several days at a temperature of 3–5°C. The effect of salt on the precipitation was, therefore, investigated.

Increasing the ammonium sulfate concentration of a 5% protein solution at pH 3.0 up to 1.5 *M* resulted in precipitation of a relatively small amount of protein which contained most of the color-producing factor. Table I gives the results obtained from a subsequent experiment which was carried out as follows: Four 10-ml. aliquots of a 5.5% salt-free solution of the albumins originally precipitated from egg white by 2.3–2.6 *M* ammonium sulfate were adjusted to pH 3.0 with  $N H_2SO_4$ . Saturated ammonium sulfate was added to 1.0, 1.5 and 2.0 *M* concentration and the pH again adjusted to pH 3.0. After two hours at room temperature the precipitates were centrifuged off. These were dissolved at pH 8.9 in a mixed buffer, 1.0 *M* with respect to boric acid and 0.25 *M* with respect to phosphate. The volume of each solution of precipitate and of each filtrate was made up to 40 ml. with the same buffer. Transmissions of 10-ml. aliquots of these solutions were read both with and without addition of ferrous iron in a Coleman spectrophotometer at 450 m $\mu$ . Duplicate 5-ml. aliquots of each solution and of the ammonium sulfate-free control were dialyzed until salt-free, dried and weighed. The color intensities and average solid contents are recorded in Table I.

The data in Table I show that, at pH 3.0, 1.5 *M* ammonium sulfate produces essentially quantitative precipitation of the Fe-binding protein from the albumin fraction of egg white and effects a five-fold purification of the active material. This fraction has been prepared repeatedly with similar results. Microbiological and colorimetric assay by Schade and coworkers gave results in agreement with our comparative colorimetric analysis. They found the pH 3, 1.5 *M*  $(NH_4)_2SO_4$ -

<sup>2</sup> Longsworth *et al.* (3) stated that the details of the method of preparation would be reported elsewhere. This information has not been reported to our knowledge.

insoluble fraction to be 8-9 times as active as untreated egg white, while the pH 3, 1.5 *M* soluble material was less than 5% as active as the insoluble fraction. These results agree with the solids data which show the concentrate contains about 10% of the dry egg white solids.

TABLE I

*Effect of  $(\text{NH}_4)_2\text{SO}_4$  Concentration on Precipitation of Iron-Binding Protein at pH 3.0*

Fraction $(\text{NH}_4)_2\text{SO}_4$ conc. in moles)	Transmission		Solids g.	Per cent
	Without Fe Per cent	With Fe Per cent		
Control	91	68	0.552	100
1.0 Precipitate	92	69	0.082	15
1.0 Filtrate	93	82	0.470	85
1.5 Precipitate	90	68	0.096	17
1.5 Filtrate	97	89	0.456	82
2.0 Precipitate	90	67	0.432	78
2.0 Filtrate	95	91	0.120	22

In an attempt to obtain material of higher specific activity, the pH 3.0, 1.5 *M* ammonium sulfate precipitate was fractionally precipitated with ammonium sulfate. Three grams were dissolved in 200 ml. of 2.0 *M*  $(\text{NH}_4)_2\text{SO}_4$  containing 0.1 *M*, pH 5.6, phosphate buffer. The  $(\text{NH}_4)_2\text{SO}_4$  concentration was raised to 2.3 *M* by dialyzing the protein solution against 23 cc. of 4 *M*  $(\text{NH}_4)_2\text{SO}_4$  in a rotating cellophane bag at 4°C.

The precipitate was collected by centrifuging, washed with 2.3 *M*  $(\text{NH}_4)_2\text{SO}_4$  solution, dissolved in water and dialyzed against running de-ionized water until salt-free. The small amount of precipitate which formed was separated, and both fractions were dried by lyophilization. In like manner, the ammonium sulfate concentration was raised successively to 2.42, 2.53 and 2.7 *M*, and the precipitates isolated in the salt-free dry form.

The lyophilized fractions were tested for color-producing activity as follows: Twenty mg. of each fraction were dissolved in 10 ml. of a buffer, pH 8.9, 1.0 *M* with respect to borate and 0.25 *M* with respect to phosphate. Each of the solutions was treated with 1.2 ml. of a solution containing 240  $\gamma$  ferrous iron. The transmission was read after standing 2 hours. The results (Table II) show no increase in specific activity of any fraction, indicating a fair degree of homogeneity.

A preparation of the concentrate, twice precipitated at pH 3.0 with 1.5 *M* ammonium sulfate, was analyzed electrophoretically in the Tiselius apparatus. The analysis was carried out at pH 4.52, ionic strength 0.1, in a buffer 0.1 and 0.15 *M* with respect to sodium acetate and acetic acid, respectively. This medium is that selected by Longworth *et al.* (3) as best adapted for electrophoretic differentiation of the principal egg white proteins.

TABLE II

*Ammonium Sulfate Fractionation of Iron-Binding Protein Concentrate*

Fraction	Weight g.	Transmission Per cent
Starting material	3.0	63
2.3 M $(\text{NH}_4)_2\text{SO}_4$ ppt. ( $\text{H}_2\text{O}$ Sol.)	1.10	61
$(\text{NH}_4)_2\text{SO}_4$ ppt. ( $\text{H}_2\text{O}$ Insol.)	0.32	62
2.42 $(\text{NH}_4)_2\text{SO}_4$ ppt.	0.43	60
2.53 $(\text{NH}_4)_2\text{SO}_4$ ppt.	0.52	62
2.70 $(\text{NH}_4)_2\text{SO}_4$ ppt.	0.31	61
2.70 $(\text{NH}_4)_2\text{SO}_4$ filtrate	0.1	—

A little more than half of the material had a falling mobility of 3.0 units; a little less than half had a mobility of 3.7 units. These mobilities correspond with those reported by Longsworth (2.89 and 3.71 at pH 4.64) for the normal form of conalbumin and conalbumin of higher sedimentation constant, perhaps polymeric, stable at pH values below 4, and slowly reverting to the electrophoretically normal form at higher pH values. Traces of material (together probably less than 5% of the whole) having mobilities approximately those of globulin  $G_2$  and ovalbumin were present.

The results of the electrophoretic analysis showed, therefore, that the iron-binding concentrate consisted essentially of pure conalbumin with traces of globulin  $G_2$  and ovalbumin. Since other tests had shown ovalbumin and globulin  $G_2$  to be inactive, the only component remaining which was resolved electrophoretically is conalbumin. Either conalbumin and the iron-binding protein are identical or the iron-binding component is present in such small amounts (<2%) that it was not resolved. The latter hypothesis is improbable, since, on fractionation of the preparation by salt and by partial adsorption procedures, no change in the specific activity of any of the fractions was observed. The amount of protein isolated by the method described is approximately 10% of the egg white solids. This yield is in fair agreement with the conalbumin concentration as given by Longsworth *et al.* (3).

The only protein of the egg white which has not been specifically eliminated from consideration by actual test is the ovomucoid. However, the essentially quantitative concentration of the active principle in the conalbumin preparation which is electrophoretically free of ovomucoid is ample evidence that ovomucoid is not the substance in question.

## SUMMARY

1. The iron-binding protein of egg white has been concentrated in a fraction which has been shown electrophoretically to be at least 95% conalbumin. Approximately 80% of the conalbumin of egg white was isolated in this fraction.

2. The antibacterial protein and conalbumin appear to be identical.

3. Crystalline egg albumin, crystalline lysozyme and the globulin fraction of egg white are inactive in producing bacterial inhibition by iron deprivation.

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# Hydrolysis of Soybean Oil Meal Proteins by Some Proteolytic Enzymes\*

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## INTRODUCTION

A study of the hydrolysis of the proteins of soybean oil meal by proteolytic enzymes, and of the effect of autoclaving the soybean oil meal on the extent of proteolytic hydrolysis, is of interest because of the influence of heating on the growth-promoting properties of the soybean proteins for the growing chick.

Considerable research has been reported recently on the action of the proteolytic enzymes on pure proteins (1, 2, 3, 4, 5), but not much has been done on the action of proteolytic enzymes on proteins in combination with complex materials as they occur in nature. Wooley and Sebrell (6) have used combinations of pepsin, trypsin and erepsin for digesting plant and animal material in preparation for the tryptophane determination.

*In vitro* digestion studies on soybean proteins with the proteolytic enzymes are limited. These studies have been summarized by Jones (7). A substance which inhibits the activity of the trypsin has been found in raw soybean oil meal by Ham and Sandstedt (8) and Bowman (9). This was crystallized by Kunitz (10) and shown to be a protein.

Following an investigation of the influence of autoclaving soybean oil meal upon the availability of cystine and methionine for the chick (11), it appeared desirable to study the influence of autoclaving upon the digestibility of the soybean proteins. The data presented here were obtained during an investigation of *in vitro* digestion of the soybean proteins by proteolytic enzymes and during a study of methods of conducting such an investigation.

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## EXPERIMENTAL

The six soybean oil meal samples used in this study were portions of meals previously used by Evans and Mc Ginnis (11) to study the influence of autoclaving on the availability of cystine and methionine for the chick. These were a "raw" soybean oil meal and meals which had been autoclaved for 30 minutes at steam pressures required to give temperatures of 100°C., 110°C., 120°C. and 130°C., and for 60 minutes at a pressure to produce a temperature of 130°C.

Several different methods of carrying out the enzymatic digestions were tried. The method that was used for obtaining the data reported in this paper was a modification of the procedure of Wooley and Sebrell (6). A 2.0 g. sample of the soybean oil meal was extracted with ethyl ether in a Soxhlet extractor. The sample was transferred to a 200 ml. Erlenmeyer flask, and, for the digestion with pepsin, 50 ml. of 0.1 *N* HCl, 10 mg. of pepsin<sup>1</sup> (1:10,000 potency) and 10 drops of tricresol were added. The flask and contents were incubated for 48 hours at 37°C. with frequent shaking. For digestion with trypsin, either initially or after pepsin digestion, the solution was made alkaline to phenolphthalein and 1.5 g. of anhydrous K<sub>2</sub>HPO<sub>4</sub>, 50 mg. of trypsin (1:300 potency), and 10 drops of tricresol were added. The flask and contents were incubated for 48 hours at 40°C. Digestion with erepsin was carried out at the same pH as the trypsin digestion. Following digestion with trypsin, 100 mg. of erepsin were added and the flask and contents incubated for 48 hours at 40°C. Following pepsin digestion, or when erepsin only was used, the solution was made alkaline to phenolphthalein and 1.5 g. of K<sub>2</sub>HPO<sub>4</sub> and 100 mg. of erepsin were added. The flask and contents were incubated at 40°C. for 48 hours with frequent shaking. Similar results were obtained with this procedure as were obtained when the pH was carefully adjusted to 8.4 for tryptic digestion and to 7.8 for ereptic digestion. For the water digestion 2.0 g. of soybean oil meal, which had been extracted with ether, were incubated at 40°C. with 50 ml. of distilled water and 10 drops of tricresol.

Several methods of determining the extent of proteolytic digestion were used. The liberation of free amino groups was determined on the digests by the method of Van Slyke (12). These values are reported as *per cent* of total nitrogen liberated as amino groups. Total nitrogen was determined by the Kjeldahl-Gunning-Arnold method (13). Two g. samples of the soybean oil meals were hydrolyzed by heating with 20% HCl for 16 hours in an oil bath held at 125-135°C. *Per cent* of total nitrogen liberated as amino groups was determined by the Van Slyke (11) procedure.

Insoluble protein was determined by acidifying the digest to methyl red with HCl, filtering through No. 42 Whatman filter paper and washing three times with hot water. Nitrogen was determined on the residue by the Kjeldahl-Gunning-Arnold method (13).

To determine the trichloroacetic acid-precipitated nitrogen, 25 ml. of a 5% solution of trichloroacetic acid were added to the acidified digest, which was then filtered through a No. 42 Whatman filter paper and washed three times with hot water containing a little trichloroacetic acid. This fraction contained the insoluble protein as well as the soluble protein that was precipitated by trichloroacetic acid.

<sup>1</sup> The enzyme preparations were obtained from the Wilson Laboratories, Chicago, Illinois.

The filtrate from the trichloroacetic acid precipitation was made to 1 *N* with HCl, and 25 ml. of phosphotungstic acid solution (50 g. of phosphotungstic acid/500 ml.) were added. If precipitation was not complete, sufficient solution was added until no more precipitate was formed with further additions. After standing overnight in the refrigerator the precipitate was filtered through a No. 42 Whatman filter paper and washed three times with an ice cold solution containing 1 *N* HCl and 1% phosphotungstic acid. Nitrogen was determined on the precipitate by the Kjeldahl-Gunning-Arnold method (13).

Because it was felt that the extent of protein denaturation by autoclaving might be related to the action of the proteolytic enzymes on soybean oil meal, a peptization study with the meals was conducted. The proteins of the six soybean oil meals were divided into three fractions by a modification of the procedure of Lund and Sandstrom (14). Since Evans and St. John (15) found no differences in the globulin and prolamine fractions of different soybean oil meals, these fractions were not determined as such. The proteins were divided into a fraction soluble in a 5% solution of potassium chloride (albumins and globulins), a fraction insoluble in 5% potassium chloride solution, but soluble in a 0.2% potassium hydroxide solution (glutelins), and the residual protein.

## RESULTS AND DISCUSSION

The data showing amino group liberation by pepsin, trypsin, erepsin, or combinations of these enzymes are presented in Table I. Approximately 2% of the total nitrogen of the soybean oil meals was present as amino groups soluble in water. Hydrolysis with 20% HCl for 18 hours liberated amino groups equal to 74% of the total nitrogen, which

TABLE I

*The Influence of Autoclaving Soybean Oil Meal on the Liberation of Amino Nitrogen by Proteolytic Enzymes*  
(Per cent of Total Nitrogen Liberated)

Heat treatment of soybean oil meal		Enzymes used							
Time	Temp.	Pepsin	Pepsin Trypsin	Pepsin Trypsin Erepsin	Trypsin	Trypsin Erepsin	Pepsin Erepsin	Erepsin	None
min.	°C.								
None		13	30	35	15	22	25	11	4
30	100	12	33	36	27	31	27	15	3
30	110	12	33	36	31	32	28	22	2
30	120	12	33	37	30	33	29	23	2
30	130	9	31	33	29	28	26	21	2
60	130	7	23	29	25	24	18	18	2



may be less than the actual potential amino groups due to loss of amino nitrogen in the humin formed. The greatest liberation of amino groups by enzymes was 37% of the total nitrogen by the pepsin, trypsin and erepsin digest of the meal which had been autoclaved at 120°C. for 30 minutes.

Table II presents the values for the undigested protein following pepsin or trypsin digestion, both the insoluble protein and that precipitated by trichloroacetic acid. Quantitative results were not obtained with the phosphotungstic acid precipitation. However, some interesting qualitative observations were made. The addition of 25

TABLE II  
*The Influence of Autoclaving Soybean Oil Meal on the Per Cent of Total Protein Not Digested by Proteolytic Enzymes*

Heat treatment of soybean oil meal		Enzyme used					
		Pepsin		Trypsin		Pepsin, trypsin, erepsin	None
		Insoluble Protein	Trichloroacetic acid ppt.	Insoluble Protein	Trichloroacetic acid ppt.	Trichloroacetic acid ppt.	Water insoluble protein
Time	Temp.						
min.	°C.	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
None		13	13	9	48	13	34
30	100	17	17	7	25	9	79
30	110	17	17	7	18	10	89
30	120	19	20	6	16	13	92
30	130	25	26	8	15	13	91
60	130	37	39	15	20	23	89

ml. of phosphotungstic acid to the trichloroacetic acid filtrate of the pepsin digest gave a flocculent precipitate that filled a 250 ml. beaker about half full. No additional precipitate was obtained with more phosphotungstic acid. Fifty ml. of phosphotungstic acid was required for complete precipitation when trypsin was used as an enzyme, although the bulk of the precipitate was very much smaller and finer than with the pepsin digest which indicates a smaller molecular size for the trypsin than the pepsin digest. This agrees with the statement of Sumner and Somers (16) that pepsin hydrolyzes proteins to proteoses and peptones, while trypsin hydrolyzes the proteoses and peptones to smaller particles.

The ability of trypsin to liberate amino groups was doubled by autoclaving the raw soybean oil meal at 110°C. for 30 minutes (Table I). It was also doubled when preceded by pepsin digestion.

Trypsin digestion was more effective than pepsin in solubilizing the soybean oil meal proteins (Table II), apparently acting upon much of the insoluble protein that was not hydrolyzed by pepsin. Trypsin did not act on a large amount of the soluble protein that was hydrolyzed by digestion with pepsin (Table II). Autoclaving raw soybean oil meal decreased the protein precipitated by trichloroacetic acid after trypsin digestion and caused a partial denaturation of the proteins as indicated by decreased solubility in potassium chloride or potassium hydroxide solutions (Table III).

TABLE III

*The Influence of Autoclaving on the Protein Distribution of Soybean Oil Meals*

Heat treatment of soybean oil meal		Per Cent of Total Protein Soluble in		
Temp	Time	KCl*	KOH†	Residue
°C.	min	Per cent	Per cent	Per cent
	None	68.2	25.4	6.4
100	30	27.3	49.0	23.7
110	30	10.2	56.7	33.1
120	30	7.8	36.5	55.7
130	30	8.6	18.8	72.6
130	60	9.8	7.0	83.2

\* A 5% solution of KCl was used. This fraction includes the albumins and the globulins.

† A 0.2% solution of KOH was used. This fraction includes the glutelins.

The greater action of trypsin on the autoclaved than on the raw soybean oil meal might be due to trypsin not being able to hydrolyze some native soybean proteins but being able to hydrolyze them after being denatured or partially hydrolyzed by pepsin (16) or it might be due to an inhibition of trypsin activity by the trypsin inhibitor of Ham and Sandstedt (8) and Bowman (9) which was destroyed by autoclaving or digestion with pepsin.

Digestion of the soybean oil meal proteins with trypsin alone carried the hydrolysis of the proteins farther than digestion with pepsin as evidenced by the greater number of amino groups liberated (Table I)

and the smaller molecular size of the fraction precipitated by phosphotungstic acid. Digestion with both pepsin and trypsin gave a greater number of liberated amino groups than digestion with either one alone.

Erepsin, a preparation of intestinal juice enzymes, appears to have some tryptic activity as well as the peptidase activity. The use of erepsin after pepsin and/or trypsin digestion increased the amino groups' liberation in nearly every case.

Autoclaving at temperatures up to 120°C. for 30 minutes had little effect on the ability of pepsin, alone or in any combination, to liberate amino groups or to hydrolyze the intact proteins. Autoclaving at 130°C. decreased amino group liberation and increased the amount of undigested protein in all cases.

### SUMMARY

The extent of hydrolysis of the soybean oil meal proteins by *in vitro* digestion with pepsin, trypsin, erepsin, or various combinations of these three enzymes, was determined on raw and autoclaved soybean oil meals. The autoclaving temperatures were 100°, 110°, 120° and 130°C. for 30 minutes and 130°C. for 60 minutes. The extent of denaturation of the proteins was followed by a peptization study.

Digestion with pepsin partially hydrolyzed all of the soluble proteins and part of the insoluble ones. It did not act as readily on the soybean oil meals that had been autoclaved at 130°C. as on those receiving less drastic heat treatment. The small number of amino groups liberated and the voluminous phosphotungstic acid precipitate obtained indicated proteoses and peptones to have been the digestion products.

Trypsin digestion hydrolyzed part of the soluble proteins, but acted to a greater extent on the denatured proteins and partially hydrolyzed proteins. It carried the hydrolysis further than did pepsin digestion, liberating a considerable number of amino groups and giving a less voluminous phosphotungstic acid precipitate.

Erepsin carried the hydrolysis a little further toward completion, hydrolyzing some groups that pepsin and trypsin did not. The most complete hydrolysis was obtained by digestion with a combination of pepsin, trypsin and erepsin.

Autoclaving the soybean meal at 100–120°C. increased trypsin digestion. Higher autoclaving temperatures decreased digestion by all enzymes and combinations used.

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# The Mechanism of the Antibacterial Action of Quinones and Hydroquinones\*†

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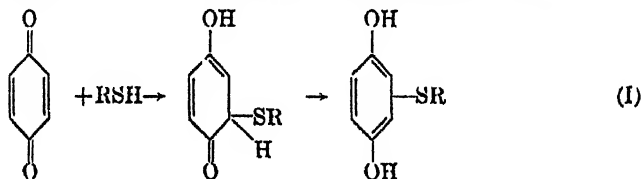
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## INTRODUCTION

In a recent paper from this laboratory (1), it was shown that clavacin and penicillic acid owe their antibacterial effects to reaction with sulfhydryl groups in the manner characteristic of  $\alpha, \beta$ -unsaturated ketones. Because *p*-quinones contain two  $\alpha, \beta$ -unsaturated ketone groupings in each molecule, this study has been extended to these compounds. While this work was in progress, Colwell and McCall (2) showed that the action of certain naphthoquinone derivatives on *E. coli* was prevented by sulfhydryl compounds. Their brief report is limited to this one organism and does not include quantitative data.

Quinones and unsaturated ketones react with sulfhydryl compounds by very similar mechanisms: 1,4 addition followed by rearrangement. Addition products resulting from reactions between quinones and sulfhydryl compounds were obtained by Troeger and Eggert (3), Posner and Lipski (4), Sharvin and Lukin (5) and Récesei (6). The nature of the reactions involved was clarified by Snell and Weissberger (7) who showed that the reaction takes place according to equation I, but is sometimes accompanied by secondary reactions. This mechanism was confirmed by Fieser (8) for



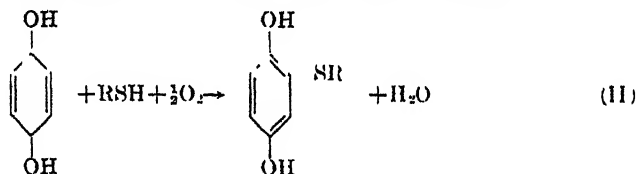
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the naphthoquinone series. Derivatives of 1,4-benzoquinone in which the 2-, 3-, 5- and 6-positions are all either occupied by substituents other than hydrogen or involved in the formation of another ring cannot react in this way. For example, vitamins K<sub>1</sub> and K<sub>2</sub> do not react with mercaptans (9).

There are several *p*-quinones among the naturally occurring antibiotics, namely, fumigatin, spinulosin, citrinin and actinomycin. Many synthetic quinones are known to be bacteriostatic (2, 10, 11, 12, 13, 14).

Hydroquinones have also been included in the present study, both because of their close chemical relationship to quinones, and because two naturally occurring antibiotic substances, gentisyl alcohol (15) and puberulic acid (16), are hydroquinones. Also, the hydroquinone corresponding to fumigatin (17, 18) occurs in nature. Other substances of this class, including hydroquinone, toluhydroquinone (13) and derivatives of *p*-aminophenol (19), have been reported to have antibacterial properties. Potter (20) has suggested that the ability of certain hydroquinones and their nitrogen analogs to inhibit enzymes may result from their reacting with sulfhydryl groups as follows:



### EXPERIMENTAL

The bacteriostatic activities of the quinones, hydroquinones, *p*-diamines and *p*-aminophenols were determined by the agar-streak method (21, 22) after the compound had been dissolved in water, alcohol or acetone. When the organic solvents were used, dilutions were chosen in such a way that no more than 0.3 ml. of alcohol or acetone was added to any plate containing 10 ml. of agar.

The effect of sulfhydryl compounds on the bacteriostatic action of quinones was determined as follows: 200 mg. of the quinone<sup>1</sup> was dissolved in a little alcohol, one

<sup>1</sup> Several of the quinones (2,6-dichloro-, 2,3,6-trichloro-, 2,6-dimethoxy-, 2,6-dichloro-3,5-dimethoxy-, and 2,6-dibromo-3,5-dimethoxyquinone), were obtained from Merck & Co.; juglone was supplied by the Wm. S. Merrill Co.; and 2,3-dichloro-1,4-naphthoquinone by the U. S. Rubber Co. The 2,3,5-trimethylphenol, used in synthesizing 2,3,5-trimethylquinone (23) was a product of the Shell Development Co. We wish to thank these organizations for their generosity in supplying these products. The 2,3,5,6-tetramethyl-quinone was prepared by the procedure of Kremers and Wakeman (24), and the 2-methyl-6-methoxyquinone according to Ashley (25). The remainder of the quinones and hydroquinones were Eastman Kodak Co. products.

molecular proportion of a freshly prepared, approximately 2 *N* aqueous solution of the sulfhydryl present after this time was oxidized with iodine using starch as indicator. The mixture was then diluted to 8.0 ml. and tested by the agar-streak method (21, 22). The procedure adopted for studying the effect of sulfhydryl compounds on the hydroquinones was the following: 400 mg. of the hydroquinone, or of the salt of the diamine or aminophenol, was dissolved in about 3 ml. of water. One equivalent of a solution of the sulfhydryl compound was added and the whole adjusted to pH 5.0 to 6.0 with sodium hydroxide. The solution was then diluted to 4.0 ml. with water, transferred to a 250 ml. Erlenmeyer flask and aerated by shaking the flask with a circular motion for 16 hrs. at 28°C. Any excess thiol compound present after this time was oxidized with iodine, using starch as indicator, and the mixture was finally diluted to 8.0 ml. and tested.

## RESULTS AND CONCLUSIONS

### A. Quinones

The data presented in Table 1 show that all the quinones studied are strongly bacteriostatic to the gram-positive organisms *Staphylo-*

TABLE I  
*Antibacterial Properties of Quinones*

Substance	Dilution units/mg. against			
	<i>E. coli</i>	<i>A. aerogenes</i>	<i>S. aureus</i>	<i>B. subtilis</i>
Quinone	4	4	200	200
2-Methylquinone	8	6	200	300
2,6-Dimethylquinone	12	12	300	400
2,3,5-Trimethylquinone	3	2	120	120
2,3,5,6-Tetramethylquinone	<1	<1	200	200
2,6-Dichloroquinone	1	2	400	300
2,3,5-Trichloroquinone	1	1	80	120
2,3,5,6-Tetrachloroquinone	<1	<1	20	10
2-Methyl-6-methoxyquinone	4	4	400	400
2,6-Dimethoxyquinone	5	5	200	750
2,6-Dichloro-3,5-dimethoxyquinone	<1	<1	12	12
2,6-Dibromo-3,5-dimethoxyquinone	<1	<1	12	12
1,4-Naphthoquinone	20	20	360	120
2-Methyl-1,4-naphthoquinone	8	8	1000	1000
2-Methyl-3-hydroxy-1,4-naphthoquinone	1	1	40	40
2-Methyl-8-hydroxy-1,4-naphthoquinone	12	12	400	400
2,3-Dichloro-1,4-naphthoquinone	2	2	100	100
Fumigatin	1	—	200	40
Citrinin	1	1	40	40



*coccus aureus* and *Bacillus subtilis*, and usually show a potency of 100 or more dilution units<sup>2</sup>/mg. They are, in general, less active against the gram-negative *Escherichia coli* and *Aerobacter aerogenes*. With the gram-negative bacteria there appears to be a relationship between chemical structure and bacteriostatic potency. Derivatives of 1,4-benzoquinone in which the 2-, 3-, 5- and 6- positions are all occupied by substituents other than hydrogen show little activity---2 units or less/mg.--against the gram-negative bacteria. Derivatives of 1,4-naphthoquinone in which the 2- and 3-positions are substituted, are likewise inactive toward the gram-negative forms. For example, benzoquinone and its mono-, di- and tri-methyl derivatives strongly inhibit *E. coli* and *A. aerogenes*, whereas tetramethyl quinone shows no detectable activity against these bacteria. This conclusion is likewise borne out by the members of the naphthoquinone series that were studied. For example, juglone (2-methyl-8-hydroxynaphthoquinone) is very active against the gram-negative organisms, whereas the isomeric phthiocol (2-methyl-3-hydroxynaphthoquinone) has but little action. The halogenated benzoquinones show a similar effect but in a less striking manner, probably because of their limited solubility.

Because activity of quinones against gram-negative organisms seems to require the presence of a free position *ortho* to a carbonyl group, it is probable that a reaction like that of equation I is involved. Hence, the effect of sulfhydryl compounds upon the bacteriostatic properties of quinones was studied, the results being presented in Table II. These data show that the activity of the quinones on the gram-negative bacteria was diminished by the mercaptans. Only one equivalent of the sulfhydryl compound was required to suppress almost completely the activity of the quinones against *E. coli* or *A. aerogenes*.

The action of quinones on gram-positive bacteria was not greatly affected by treatment with monothioglycol, but in certain instances (e.g., 2-methyl-6-methoxyquinone or 2-methylnaphthoquinone) thioglycolate or cysteine caused a diminution in activity. This result is probably due to secondary reactions which led to cyclized products such as those described by Snell and Weissberger (7).

Experiments were also carried out under similar conditions using one of the amino compounds, methylamine, glycine or asparagine, instead of the sulfhydryl compound. The amino compounds produced

<sup>2</sup> A dilution unit is that amount of material which when added to 1.0 ml. of agar, just inhibits a test organism (22).

TABLE II

*Effect of Sulfhydryl Compounds on the Antibacterial Properties of Quinones*

Quinone	Sulfhydryl compound	Dilution units/mg. against			
		<i>E. coli</i>	<i>A. aureo-genes</i>	<i>S. aureus</i>	<i>B. subtilis</i>
2-Methylquinone	None	8	6	200	200
	Monothioglycol	2	<1	200	400
	Thioglycolate	1	1	400	400
	Cysteine	2	2	200	200
2,5-Dimethylquinone	None	12	12	300	300
	Monothioglycol	<1	<1	400	400
	Thioglycolate	1	<1	80	80
	Cysteine	8	5	400	400
2-Methyl-6-methoxy-quinone	None	4	4	400	400
	Monothioglycol	3	1	400	400
	Thioglycolate	1	1	80	80
	Cysteine	1	1	80	100
Naphthoquinone	None	20	20	400	400
	Monothioglycol	6	6	400	400
	Thioglycolate	2	2	400	400
	Cysteine	4	<1	400	400
2-Methylnaphtho-quinone	None	8	8	1,200	1,200
	Monothioglycol	<1	<1	800	1,200
	Thioglycolate	1	1	100	100
	Cysteine	1	1	400	200
2-Methyl-8-hydroxy-1,4-naphthoquinone	None	12	12	400	400
	Monothioglycol	2	2	400	200
	Thioglycolate	1	1	400	120
	Cysteine	1	1	120	80

little or no inactivation of the quinones, although it was apparent, from the fact that brown or purplish colors are often produced, that a reaction of some sort takes place.

### *B. Hydroquinones*

The bacteriostatic properties of the hydroquinones and their nitrogen analogs are presented in Table III. All of the compounds have

greater activity against the gram-positive bacteria (*S. aureus* and *B. subtilis*) than against the gram-negative organisms (*E. coli* and *A. aerogenes*). Furthermore, the compounds do not differ greatly in their action on the gram-positive bacteria. With the gram-negative forms far greater differences are observed, and these are brought out particularly well when the activity per millimol is considered.

The compounds containing nitrogen were found to be more active than their oxygen analogs, and the presence of methyl groups attached to the nitrogen atoms further enhances their action. Potter (20) and

TABLE III  
*The Bacteriostatic Action of Hydroquinones*

Compound	Dilution units per mg (or per mmol) against			
	<i>E. coli</i>	<i>A. aerogenes</i>	<i>S. aureus</i>	<i>B. subtilis</i>
Hydroquinone	2 (220) <sup>a</sup>	2	200	300
Tolhydroquinone	10 (1240)	10	200	600
<i>p</i> -Aminophenol	8 (870)	15	200	250
<i>p</i> -Methylaminophenol	15 (1850)	20	300	300
<i>p</i> -Dimethylaminophenol	20 (2800)	20	250	250
<i>p</i> -Phenylenediamine	4 (430)	8	200	200
<i>p</i> -Aminomethylaniline	30 (3900)	25	60	300
<i>p</i> -Aminodimethylaniline	20 (2900)	25	200	250
<i>s</i> -Dimethyl- <i>p</i> -phenylenediamine	30 (4400)	30	250	300
Tetramethyl- <i>p</i> -phenylenediamine <sup>b</sup>	30 (4900)	30	600	600
<i>p</i> -Acetylaminophenol	<1	<1	<1	<1
Diacetyl- <i>p</i> -phenylenediamine	<1	<1	<1	<1

<sup>a</sup> Values given in parentheses represent dilution units/mmol.

<sup>b</sup> After oxidation with 1.0 equivalent of bromine.

Kensler, Young and Rhoads (26) have observed that these same structural changes are accompanied by increased ability of the compounds to inhibit several respiratory enzymes, and have pointed out that these are the changes which Michaelis, Schubert and Granick (27) found to favor stability of the corresponding semiquinone free radicals. Furthermore, it was found that preliminary oxidation of the compounds to the semiquinone stage with one equivalent of bromine led to no change in activity. Oxidation to the quinone stage with two equivalents led to diminished activity, presumably because the imino-

TABLE IV

*Effect of Aeration in the Presence of Sulfhydryl Compounds on  
Bacteriostasis by Hydroquinones*

Hydroquinone	Sulfhydryl compound	<i>E. coli</i>	<i>A. aerogenes</i>	<i>S. aureus</i>	<i>B. subtilis</i>
Toluhydroquinone	None	20	16	600	> 600
	Monothioglycol	1	1	120	120
	Thioglycolate	4	2	600	> 600
	Cysteine	2	2	200	600
<i>p</i> -Aminopheol	None	8	15	80	250
	Monothioglycol	3	1	80	80
	Thioglycolate	3	4	100	100
	Cysteine	3	<1	25	50
<i>p</i> -Methylaminophenol	None	15	15	300	300
	Monothioglycol	2	2	300	300
	Thioglycolate	2	2	300	300
	Cysteine	2	2	80	80
<i>p</i> -Dimethylamino-phenol	None	8	8	250	250
	Monothioglycol	3	1	500	500
	Thioglycolate	1	1	80	800
	Cysteine	1	1	20	20
<i>p</i> -Aminomethylaniline	None	10	10	250	300
	Monothioglycol	3	1	160	100
	Thioglycolate	3	1	300	160
	Cysteine	2	1	160	300
<i>p</i> -Aminodimethyl-aniline	None	25	30	450	450
	Monothioglycol	2	2	150	150
	Thioglycolate	2	2	150	150
	Cysteine	1	1	60	60
<i>s</i> -Dimethyl- <i>p</i> -phenylenediamine	None	8	8	160	160
	Monothioglycol	2	4	160	160
	Thioglycolate	2	1	80	160
	Cysteine	2	2	160	160
Tetramethyl- <i>p</i> -phenylenediamine	None	8	6	200	200
	Monothioglycol	6	6	200	200
	Thioglycolate	4	4	200	200
	Cysteine	2	2	60	60

quinones first formed decompose spontaneously to benzoquinone. Also, the acetylated compounds *p*-acetylaminophenol and diacetyl-*p*-phenylenediamine, which do not form semiquinone radicals, are not bacteriostatic. These facts support the hypothesis that the active inhibitory substance for bacteria, as for enzymes, is the semiquinone free radical.

Certain enzymes have been shown to be protected against inhibition by hydroquinones by the presence of cysteine (20, 26). Therefore the effectiveness of sulfhydryl compounds in preventing bacteriostasis by hydroquinones was investigated. Preliminary experiments showed that sulfhydryl compounds did not interfere with bacteriostasis by hydroquinones if both were simply mixed and added to agar, which was then streaked with the test organisms. But if a mixture of the sulfhydryl compound and hydroquinone was aerated and then tested, the product had a much weaker action against the gram-negative bacteria than the untreated hydroquinone, as the data of Table IV show. The reaction products formed by monothioglycol with the hydroquinones had a less marked effect on the gram-negative *E. coli* and *A. aerogenes*, while with the gram-positive organisms, the reaction products were generally as strongly inhibitory as the hydroquinones. Cysteine and thioglycolate were found to behave in much the same way as monothioglycol. Control experiments showed that aeration in the presence of any sulfhydryl compound did not affect the bacteriostatic properties of the hydroquinones.

Experiments designed to determine whether amino compounds had an effect like that of the sulfhydryl compounds were also carried out. Aeration in the presence of methylamine, glycine and asparagine had no effect upon bacteriostasis by hydroquinones.

Since some of the compounds are phenols, it is important to mention that the bacteriostatic powers of typical phenols are exhibited only at much higher concentrations. By the agar-streak method phenol and resorcinol show less than one dilution unit/mg. against the test organisms.

## SUMMARY

1. Quinones are generally less active against the gram-negative than against the gram-positive bacteria. The activity of quinones against the gram-negative organism is associated with the presence of one or more unsubstituted positions *ortho* to carbonyl groups.

2. All quinones tested were highly active against gram-positive bacteria and no structural requirement was apparent.

3. Monothioglycol, thioglycolate and cysteine prevent the inhibition of growth of gram-negative bacteria by quinones. The action of quinones against the gram-positive organisms is not prevented by sulfhydryl compounds.

4. Hydroquinones and their nitrogen analogs are bacteriostatic to both gram-positive and gram-negative bacteria

5. Hydroquinones arranged in the order of their effectiveness against gram-negative bacteria are found to fall in the order of increasing stability of the corresponding semiquinone free radicals.

6. The action of hydroquinones against gram-negative bacteria is prevented by aeration in the presence of monothioglycol, thioglycolate or cysteine.

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## Further Studies on *in vivo* Tooth Decalcification by Acid Beverages\*

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### INTRODUCTION

It was shown by McClure (1) in 1943 that various acid beverages and fruit juices, when ingested by white rats, had a deleterious effect on the teeth. We have corroborated these findings (2, 3) and have shown that similar effects are produced by a number of acids on the teeth of rats, hamsters and puppies. The acids studied were phosphoric, sulfuric, citric and lactic acids. The addition of small amounts of fluorine to these acid solutions afforded partial protection from tooth decalcification.

This work has been extended to include (a) the effects on rats' teeth of drinking acetic acid (pH 2.6), orange juice, and phosphoric and lactic acids at pH 4.5, and (b) the effects of phosphoric and citric acid solutions with and without fluorine on the permanent teeth and tissues of dogs. The results of an exploratory study with two monkeys drinking phosphoric and citric acid solutions are also presented.

### EXPERIMENTS AND RESULTS

#### *Rat Studies*

The procedure was the same as that in previous studies. Young white rats, caged separately, were given 20 ml. daily of the test solutions, each containing 10% sucrose, for 1-2 weeks. A commercial dog food<sup>1</sup> was fed *ad libitum*. At the end of the experimental period the animals were sacrificed, the jaws removed and the destruction of the teeth judged according to the scoring plan published previously (4).

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\* The opinions and views set forth in this article are those of the writers and are not to be considered as reflecting the policies of the Navy Department.

<sup>1</sup> G.L.F. dog food from Canandaigua, New York.



Since acetic acid is commonly encountered in pickled foods, *etc.*, ten rats were given a 4% solution of this acid (pH 2.6), corresponding in strength to vinegar. Inasmuch as the animals would consume an average of only 10 ml. daily, half of the rats were killed after one week and the rest after two weeks. It can be seen (Table I) that after

TABLE I

*The Effect on Rats' Molars of Drinking 20 ml. Daily of Various Acid-Sucrose Solutions*

Solution	pH	No. of rats	Average molar scores <sup>1</sup>			
			One Week		Two Weeks	
			Upper jaw	Lower jaw	Upper jaw	Lower jaw
Acetic acid†						
4.0%	2.6	10	0.6	2.7	0.9	4.1
Orange juice	3.7	10	0.5	4.3	2.0	5.6
Citric acid†						
0.2%	2.6	16	1.4	3.9		-
Phosphoric acid						
0.055%-plus NaOH	4.5	12			0.0	0.0
Lactic acid						
0.43%-plus NaOH	4.5	8	-	-	0.0	0.0

\* A score of zero corresponds to no detectible acid effect; a score of 6 represents almost complete destruction of lingual enamel with some destruction on other surfaces. See reference (4).

† Average daily consumption, 10 ml. per rat.

‡ Data from (3).

one week of drinking this solution only mild etching of the rats' molars was evident, in line with the low level of consumption. After two weeks, however, enamel destruction was equal to that reported previously (3) for a citric acid solution of the same pH. The effect was predominantly on the lower molars.

Citrate ion is known to have a strong affinity for calcium and apparently is an additional factor in the tooth decalcifying properties of citrus fruits and juices (5). Accordingly, the effects of offering a fresh-frozen, canned orange juice (pH 3.7) to ten rats were determined. After one week, the rats' teeth showed a degree of destruction comparable to that obtained previously (3) with 0.2% citric acid solution (Table I). After two weeks, severe erosion of the enamel of the mandibular molars was evident.

Since some foods (*e.g.*, buttermilk) have pH values of about 4.5, it was considered of interest to determine whether etching of the teeth would result from drinking phosphoric and lactic acid solutions at this higher pH level. Accordingly, twelve rats were given a 0.055% phosphoric acid solution, and eight rats were given a 0.43% lactic acid solution. Both solutions were adjusted to pH 4.5 by the addition of NaOH. After two weeks' drinking, no etching of the molars could be detected in any of the animals (Table I).

*Dog Studies*

(a) *Phosphoric acid.* It was reported earlier (2) that limited amounts of a cola-type beverage produced etching of the deciduous teeth of puppies, and that fluorine in the liquid was effective in reducing the tooth erosion. These same dogs were continued on the experiment to note the effects of the acid solutions, with and without fluorine, on the permanent teeth. The concentrations of fluorine in various tissues of these dogs was also determined.

The test solution was composed of 0.055% phosphoric acid and 10% sucrose, pH 2.6. Of six litter-mates, two puppies served as controls, one drinking water and one 10% sucrose solution, two others were given the acid-sucrose solution, and two were given the same solution but with 1 and 20 ppm. of fluorine (as NaF) added. Five hundred ml. of each solution were allowed daily, 350 ml. in the morning and 150 ml. in the afternoon. A commercial dog food<sup>2</sup> containing 35 ppm. fluorine was fed twice daily on a body weight basis. For the first two months the ground feed was mixed with an equal weight of evaporated milk; subsequently it was mixed with water.

The dogs were started on the experiment at ten weeks of age. As the deciduous teeth loosened they were extracted and examined. After 130 days on the experiment, the animals were sacrificed, the heads autoclaved to permit easier removal of soft tissues, and the permanent teeth examined grossly and under the low-power microscope.

Comparison of the teeth of the dogs drinking the phosphoric acid solution with those of the control dogs revealed a moderate etching of the enamel comparable to that previously found on the deciduous teeth. Decalcification was sufficient to produce definite ridging at the gingival margin. The effect was most prominent on the labial surfaces of the anterior teeth, and on the lingual surfaces of the posterior teeth. Generally the lower teeth were more affected than the upper.

The teeth of the dogs drinking fluoride-containing solutions definitely showed less decalcification. They had, however, a more smoothly polished surface than those of the controls, indicative of mild acid attack. The higher fluorine level (20 ppm.) was more protective than the lower level (1 ppm.).

Comparing the same incisor from different dogs with an impacted tooth from one of the animals (Fig. 1), it is seen that abrasion occurred on all the erupted teeth. However, only in the dogs receiving the acid solution alone and the one getting but 1 ppm. of fluorine did the cusps wear through the enamel layer. The dissolution of enamel caused by acid ingestion is most clearly evident on the labial surfaces; in this connection the decreased effect of the acid in the presence of fluoride, especially the higher level studied, is evident.

<sup>2</sup> (I.L.F. dog food from Canandaigua, New York.

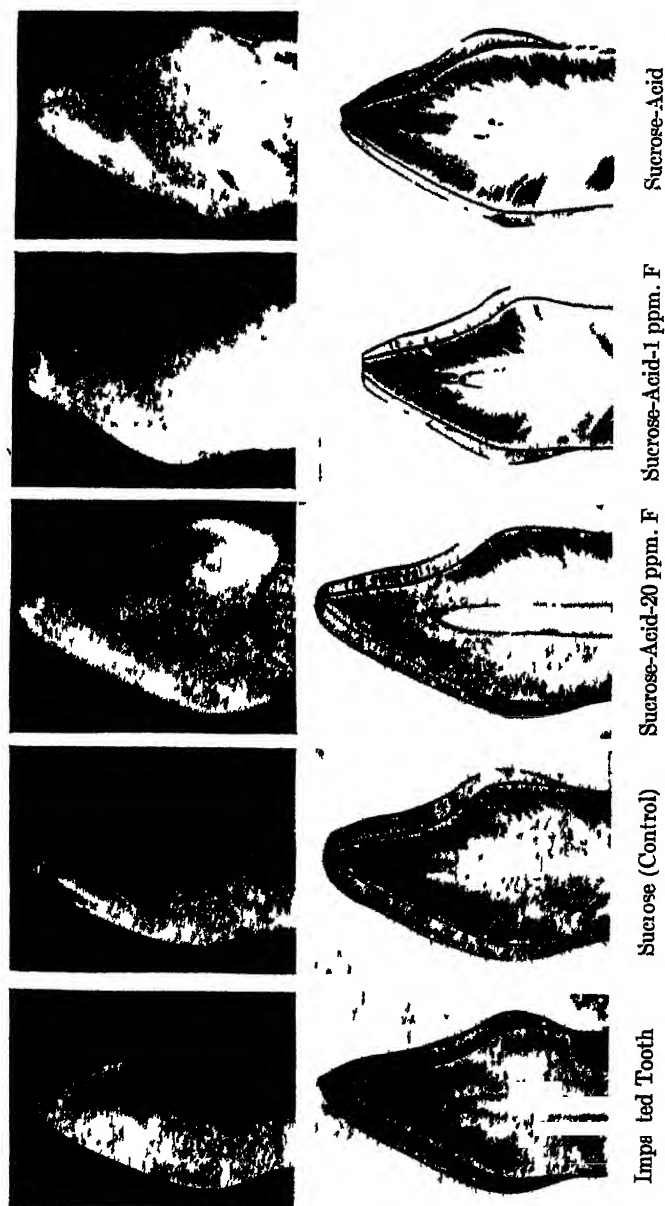


FIG. 1.

Photomicrographs of intact mandibular incisors (side view) and ground sections of same from litter-mate dogs allowed 500 ml. daily of different solutions for 130 days. All solutions contained 10% sucrose; the acid solutions (pH 2.6) contained .055% phosphoric acid. The labial surface is to the left in each picture.

At autopsy, samples of liver, muscle (biceps) and bone (femur) were taken from each animal for fluorine analyses. After weighing, the tissues were oven dried at 100°C., ground and stored in air-tight containers. Fluorine analyses were performed according to the A.O.A.C. method (5). The results (Table II) show that the dog drink-

TABLE II

*Fluorine Content of Tissues of Dogs Ingesting Acid-Sucrose-Sodium Fluoride Solutions*  
(Values in ppm. F of fresh tissue)

First Experiment				
Dog No	Solution Ingested	Femur	Liver	Muscle
6	Water	134	0.2	0.8
1	Sucrose (10%)	132	0.3	2.0
2	Phosphoric-sucrose	148	0.1	0.9
3	Phosphoric-sucrose	140	0.1	1.2
5	Phosphoric-sucrose with 1 ppm. F	192	0.3	1.0
4	Phosphoric-sucrose with 20 ppm. F	298	0.2	0.6
Second Experiment				
7	Sucrose (10%)	188		
8	Citric-sucrose	146		
9	Citric-sucrose with 1 ppm. F	174		
10	Citric-sucrose with 20 ppm. F	320		

ing a solution with 20 ppm. of fluorine deposited more fluorine in the bone than did the animals drinking the non-fluoride solutions. The differences in fluorine content of the livers and muscles are not significant.

(b) *Citric acid.* The second study involved four litter-mate puppies. One dog received a Navy-issue synthetic lemonade (pH 2.6), two others were fed the same solution containing 1 and 20 ppm. of fluorine (as NaF), and the fourth, serving as a control, received a 10% sucrose solution. The feed was the same as in the preceding experiment. Since all of the animals consistently refused the acid beverage, the daily allowance was reduced to 150 ml. At times not even this volume was consumed. The experiment was continued for 137 days. As the deciduous teeth loosened they were extracted, attempts being made to extract teeth from all dogs on the same days. At the end of the experiment the dogs were sacrificed and the teeth examined. Bone samples were taken for analyses as described above.

The effects of the various solutions on the dogs' teeth were not as clearly defined as in the first experiment because of the animals' inconsistent consumption of the test solutions. Both the deciduous and permanent teeth of the dogs drinking the acid beverage showed distinct etching of the enamel. Definite correlation between the amount

of fluorine in the solution and the protection from etching, however, could not be made.

Fluorine analyses of bone samples (Table II) revealed a retention of fluorine similar to that by the dogs in the first experiment. The effect of 20 ppm. fluorine in the drink is readily apparent in the considerably higher bone-fluorine.

### *Monkey Studies*

Since the monkey closely resembles man in his dentition and methods of drinking, an exploratory study was made with two young rhesus monkeys weighing 5-6 pounds. Before the experiment was started, the animals were anesthetized and the opposing right deciduous central incisors extracted to serve as control teeth.

The diet was the same as that fed the stock colony: dog feed pellets, boiled eggs, carrots and an occasional apple, banana and orange. To accustom the monkeys to drinking a sweet solution, they were given 160-170 ml. daily of a 10% sucrose solution from a bottle and tube attached to each cage. After three weeks, acidic beverages were substituted for the sucrose solution. One animal was given synthetic lemonade and the other a sucrose-phosphoric acid solution, both solutions having a pH of 2.6. After 30 days on these solutions the left opposing central incisors were removed. After an additional 30 days on the solutions the monkeys were sacrificed and their teeth examined.

Gross examination of the deciduous teeth revealed that although they were severely worn down by attrition, they generally showed the "rounding off" of the abraded edges commonly noted in acid ingestion. This was most apparent in comparing the incisors extracted prior to the experiment with those extracted after the monkeys had been drinking the acid solutions for one month. This comparison also suggested that the phosphoric acid solution caused somewhat greater dissolution of the enamel than did the citric acid beverage.

### DISCUSSION

The results with acetic acid and orange juice indicate that these food acids act *in vivo* on rats' molars in a manner comparable to that noted earlier (3) for solutions of citric and lactic acids of the same pH (2.6). The observation that solutions of phosphoric and lactic acids of pH 4.5 do not etch rats' teeth is in sharp contrast to the effects of the same acids at pH 2.6. Experiments now under way suggest that *in vivo* decalcification by these acids decreases rapidly as the pH increases above 2.6, and that little effect takes place above 3.0. It is recognized, however, that the acid anion also influences enamel solubility to some

extent. In a recent study, McClure and Ruzicka (5) have shown that, even at neutral reaction, citrate ion is destructive to rats' molars.

The effect on dogs of ingesting 3 to 10 mg. of fluorine in solution daily for 4 to 5 months is apparent in the high bone-fluorine levels of the two dogs drinking solutions containing 20 ppm. of fluorine (Table II). It has been shown by Marcovitch and Stanley (7) and by Lawrenz *et al.* (8) that fluorine in solution is more readily retained by rats than fluorine in the food. Our findings with dogs would indicate a similar retention by this species. The dogs drinking fluorine solutions ingested slightly larger total quantities of fluorine than did the other dogs, although the basal ration contained considerable fluorine (35 ppm.).

In examining the jaws, areas of apparently abnormal bone porosity were observed on the external surface of the mandible of the dog receiving 20 ppm. of fluorine. This effect was particularly evident on the surface below the alveolar ridge in the area of the molars and on the ramus, but it was not noted on the maxillae. This observation is of interest in that Largent *et al.* reported (9) that rabbits fed high levels of fluorine developed gross changes in the mandible.

The observation that limited amounts of acid beverages produced enamel erosion in dogs and monkeys suggests that similar effects might be produced in man. However, the difficulties involved in studies of this nature with man are numerous. The technic of having an animal serve as its own control by extracting comparable teeth before and after an experimental period is the best known to us. Detection of mild enamel etching prior to extraction is difficult even for the trained observer, and for this reason it is not surprising that many instances of mild tooth erosion in human beings go unnoticed or unrecorded.

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#### SUMMARY

1. Drinking of an acetic acid-sucrose solution of pH 2.6 for 1-2 weeks produced mild to moderate etching of rats' molars. Orange juice of pH 3.7 decalcified the teeth severely in two weeks. Solutions of phosphoric and lactic acids of pH 4.5 caused no etching when fed for two weeks.

2. The permanent teeth of dogs drinking a phosphoric acid-sucrose solution of pH 2.6 for 130 days showed etching of the enamel similar to that found earlier on the deciduous teeth. Low levels of fluorine in the acid solution decreased the extent of tooth decalcification.

The deciduous and permanent teeth of dogs drinking citric acid-sucrose solution of pH 2.6 showed etching of the tooth enamel.

Two dogs ingesting solutions containing 20 ppm. of fluorine had bone fluorine levels considerably higher than those of control dogs receiving solutions without fluorine.

3. Consumption of 165 ml. daily for one month of phosphoric and citric acid solutions of pH 2.6 produced noticeable erosion of the deciduous teeth of young monkeys.

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# Studies on Thymonucleodepolymerase \*

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## INTRODUCTION

Most of the recent workers have followed thymonucleodepolymerase activity through measurement of decrease in viscosity of the reacting mixture (1, 2, 3, 4). The decrease in viscosity indicates a decrease in size of the molecules but gives little information regarding the nature of the products of the reaction. The present paper describes a method analogous to that of Kunitz (5) for determination of thymonucleodepolymerase activity based on the rate of liberation of acid-soluble phosphorus. Results by this method are compared with results by following the decrease in viscosity. A method for the purification of thymonucleodepolymerase is also described.<sup>1</sup>

## EXPERIMENTAL

*Method of Purification of the Enzyme.* The material used was fresh hog pancreas obtained from the slaughter house. Frozen hog pancreas was also tried and several batches were found active. However, not all the commercially obtainable frozen material was satisfactory. On the other hand, no significant loss of activity occurred during freezing of the pancreas in the laboratory refrigerator.

*Extraction.* Cooled or frozen pancreas was ground in a Waring blender with an equal volume of 0.2 N H<sub>2</sub>SO<sub>4</sub>, and was centrifuged. The liquid was collected and neutralized. We used 0.2 N H<sub>2</sub>SO<sub>4</sub> instead of 0.25 N, as originally suggested by Kunitz and Northrop (7), because hog thymonucleodepolymerase was not quite

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† The early part of this work was done in the Department of Physiological Chemistry, School of Medicine, University of Arkansas, and was assisted by Mrs. Jane Steel. In Milwaukee, it was assisted by Miss Helen Crenshaw. Their help is gratefully acknowledged. I am highly indebted to Dr. Paul L. Day, who loaned me some equipment, thus enabling me to continue the work without interruption.

<sup>1</sup> Preliminary report describing this procedure was published (6).



stable in the acid solution at room temperature. Nevertheless, the initial extraction with acid was found advantageous in several respects. It offered protection from the action of proteolytic enzymes. If the extraction was carried through rapidly enough and the temperature was not allowed to rise above 15°C., the yield from acid extraction was equal to, or higher than, that of any other extraction tried. Additional advantages were that the amount of extracted inactive protein was comparatively low, and the centrifugation was fairly easy.

The increase in potency during the extraction was considerable but this could only be judged approximately since no successful method of determining thymonucleodepolymerase in the minced tissue has been developed (see below). Thus, the initial amount of enzyme was roughly estimated on the basis of several methods of extraction. According to such estimates the increase in potency must have been about 10-fold.

*Purification with Bentonite.* The neutralized extract was treated with 7.5 g. of Panther Creek Bentonite<sup>1</sup> per 100 cc., thoroughly mixed and centrifuged. The liquid usually contained 80-90% of the original activity, while the amount of protein was reduced to about 35-45%. The potency was increased about 2.5-fold.

*Precipitation with Methyl Alcohol.* The liquid was again readjusted to pH 7, cooled in the ice bath, and treated with 2 volumes of cold methyl alcohol. The mixture was left in the ice bath for 20-30 minutes and centrifuged. The liquid was discarded and the precipitate was suspended in water (1/5 of the previous volume) and centrifuged. This step caused the potency to increase about 2.5-fold, making a total increase of about  $10 \times 2.5 \times 2.5 = 62$ -fold. At this stage, the preparation can be successfully dried and preserved. Most of the reported experiments were performed with the preparation at this stage of purification. The increase in potency during the purification procedure up to this stage is shown in the last two columns of Table I.

*Fractionation with methyl alcohol* led to a somewhat more purified enzyme but the yield in this step was less than 50%. It was accomplished by cooling the enzyme

TABLE I  
*Comparison of the Activity of Thymonucleodepolymerase  
Determined by the Two Different Methods*

Stage of purification	Exp. 32 Low Initial Value			Exp. 38 High Initial Value					
	P-units	V-units	V/P	P-units	V-units	V/P	Protein mg./cc.	P-units/ mg. pro- tein	V-units/ mg. pro- tein
A: neutralized original extract	6.0	605	99	70	1560	22	13.1	5.4	110
B: after purification with Bentonite	4.1	535	130	67	1460	22	3.9	17.2	375
C: after precipitation with methyl alcohol	32.2	2300	74	164	4200	25	4.5	36.5	935

<sup>1</sup> Supplied by Mr. Bechtner of American Colloid Co.

solution in the freezing compartment of the refrigerator, which resulted in the formation of a semi-crystalline precipitate. This precipitate was discarded. To the liquid an equal volume of methyl alcohol was added. The second precipitate was collected. It showed the increase in potency to be about 1.5-fold. Several buffers (pH 5 to 8) were tried in attempts to delimit the active fraction more sharply but no considerable improvement was secured.

*Assay for Activity.* Thymonucleic acid, prepared according to Hammarsten (8), was used as substrate. The preparation always exhibited a negative biuret reaction but usually had somewhat higher N:P ratio than the theoretical 1.66. The air-dried preparations usually showed around 7.5% phosphorus, but occasionally samples as low as 6.4% were encountered.

All tubes contained 1.0 cc. of the substrate solution (0.5 g. of thymonucleic acid, 4 millimols of  $MgSO_4$  made up to 100 cc. with 0.2 *M* borate buffer pH 7.1), and 0.6 cc. of the enzyme solution (in the same buffer). Incubation was carried out for 60 minutes at 37°C. The reaction was stopped by adding 1.0 cc. of 20% trichloroacetic acid. The precipitate of non-digested thymonucleic acid was centrifuged off and the aliquot of the liquid was analyzed for total phosphorus by the method of Martland and Robison (9). The control tubes containing only enzyme solution were incubated for an equal period of time. The substrate solution was added to the controls after the treatment with trichloroacetic acid. The difference between the experimental and the control tubes was considered due to the action of thymonucleodepolymerase.

The results shown in Fig. 1 indicate a fairly good proportionality between the amount of enzyme and the amount of the liberated acid-

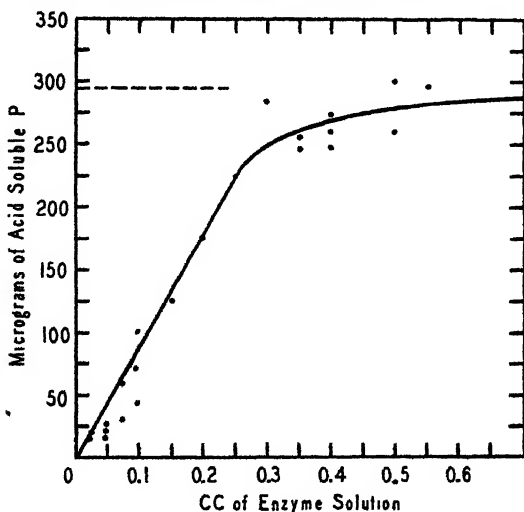


FIG. 1  
Rate of Liberation of Acid-Soluble Phosphorus  
as a Function of Enzyme Concentration

soluble phosphorus, and therefore justify this procedure for determination of thymonucleodepolymerase.<sup>3</sup>

A time-activity curve is shown in Fig. 2. The experimental conditions were identical with those of the Fig. 1, except that the amount of enzyme was constant and the time of incubation varied. Under these conditions the reaction is roughly of a zero order (a straight line up to 120 minutes). This does not agree with our previous finding (4) by the viscosity method in which a first order reaction was found. The dis-

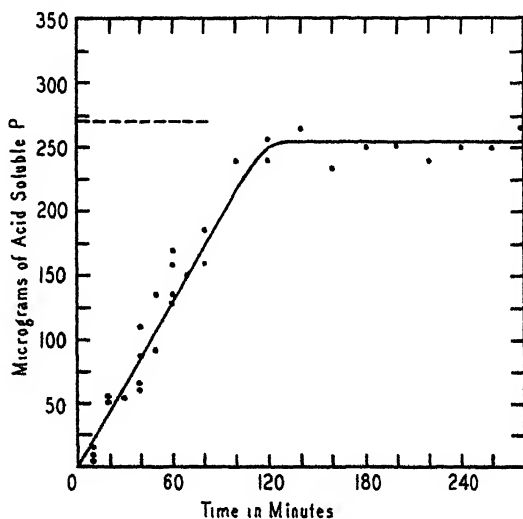


FIG. 2  
Time-Activity Relationship

crepancy is probably due to the fact that the acid-soluble phosphorus method requires 50–100 times more enzyme than the viscosity method.

Figs. 1 and 2 also show that the reaction proceeds to the end until all the phosphorus present is rendered acid-soluble. In this respect the system under investigation differs from the system composed of ribonucleic acid and ribonuclease. In the latter, Kunitz (5) found that not more than 40% of the total phosphorus is rendered acid-soluble. Zittle (10) recently confirmed the finding of Kunitz and attempted to

<sup>3</sup> The proportionality existed only with the partially purified enzymes. With extracts of the crude tissue the increased amounts of enzyme produced less than the expected increase in acid-soluble phosphorus.

explain the abrupt termination of the reaction on the basis of inhibition by the mononucleotides formed during the reaction, but came to the conclusion that such an explanation was unsatisfactory. The difference between the two systems cannot be due to the presence of phosphatase in our preparation. No phosphatase activity could be detected in the partially purified thymonucleodepolymerase, using an amount of enzyme containing 2000 viscosity units. Furthermore, we previously showed (4) that the addition of purified phosphatase to our system did not accelerate the liberation of acid-soluble phosphorus. It is of interest to point to this difference in view of the fact that the homogeneity of both types of highly polymerized nucleic acids is under discussion.

When a sufficiently large excess of enzyme was present, and the amount of substrate was varied, a straight proportionality was found between the concentration of substrate and the amount of liberated acid-soluble phosphorus (Fig. 3). In this experiment as in the previous ones, all phosphorus in thymonucleic acid was rendered acid-soluble.

For the quantitative determination of thymonucleodepolymerase, it was found more convenient to use a five-fold amount of reagents: 5 cc. of substrate solution containing 25 mg. of thymonucleic acid in 0.2 *M* borate buffer pH 7.1, made 0.04 *M* in respect to  $\text{MgSO}_4$ , and 3 cc. of the enzyme solution in borate buffer were used. One "P-unit" was defined as that amount of enzyme which, under the conditions described, will liberate 100  $\gamma$  of acid-soluble phosphorus during 60 minutes at 37°C.

The activity of thymonucleodepolymerase at different stages of purification was determined by both methods and was expressed in "V-units" (viscosity) and "P-units" (acid-soluble phosphorus). The results are shown in Table I. No close quantitative relationship between the two units could be established. The V/P ratio varied from 20 to 140, with the most common value around 50. No reasonable explanation for this variation can be offered at present. On the other hand, however, the results presented in Table I do not supply any evidence of a substantial change in the V/P ratio during the process of purification. Should the two activities represent two different enzymes a drastic change in the ratio should have been expected.

The accelerating effect of Mg ions previously found by the viscosity method (4) was confirmed by the acid-soluble phosphorus method (Table II). The maximum speed of the reaction was reached at the same level, namely, 0.025 *M*.

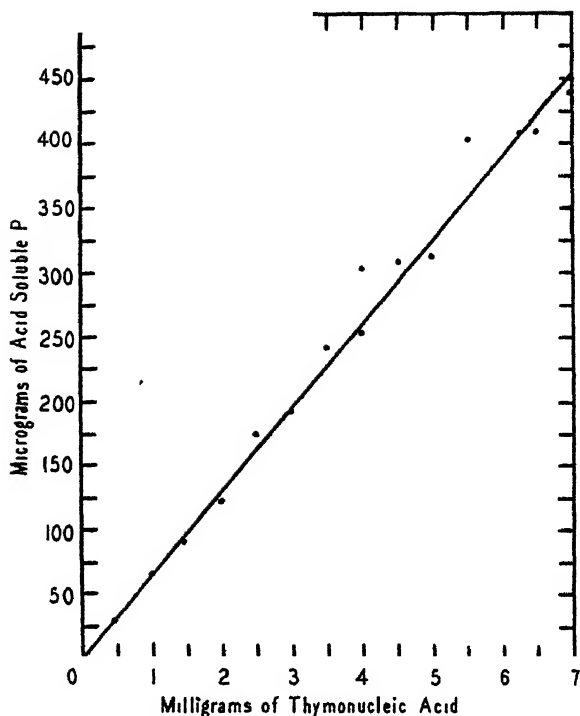


FIG. 3

Liberation of Acid-Soluble Phosphorus as a Function of the Substrate Concentration. Excess Enzyme Present

TABLE II

*Effect of Magnesium Sulfate on Thymonucleodepolymerase*

Molarity of added magnesium sulfate in the reacting mixture	$\gamma$ Acid-soluble phosphorus liberated by thymonucleodepolymerase
0.00	56
0.00125	40
0.00250	48
0.00650	178
0.0125	303
0.025	366
0.125	377
0.250	366

The inhibition by the high concentrations of neutral salts (NaCl) was also investigated by both methods and found to be similar (Table III). Finally, an experiment was made in which NaCl was added to the reacting mixture in the middle of the incubation period (tube 5,

TABLE III

*Effect of Different Concentrations of NaCl on Thymonucleodepolymerase*

Molarity of added NaCl	0.0	0.5	1.0	2.0
P-units/sample	12.55	4.52	1.08	0.16
Per cent inhibition	0.0	64	91	99
V-units/sample	11.4	3.54	1.81	0.50
Per cent inhibition	0.0	69	83	95
V/P	0.90	0.78	0.59	0.30

Table IV). In this case, the liberation of the acid-soluble phosphorus was inhibited as completely as in the control (tube 1).

Taking into consideration the evidence presented, it seems reasonable to conclude that one enzyme, provisionally called thymonucleodepolymerase, hydrolyzes thymonucleic acid at least to such a degree that the high viscosity value disappears and all phosphorus is changed to an acid-soluble form.

TABLE IV

*Effect of Addition of NaCl in the Middle of Incubation Period*

Tube No.		$\gamma$ Acid-soluble P
1	No NaCl added, incubation 30 min.	300
2	No NaCl added, incubation 60 min.	585
3	2 M NaCl, incubation 30 min.	109
4	2 M NaCl, incubation 60 min.	113
5	2 M NaCl added after 30 min. incubation, and incubated for the next 30 min.	280

## SUMMARY

A method for the purification of thymonucleodepolymerase has been described. This consists in extraction of hog pancreas with 0.2 N  $H_2SO_4$ , centrifuging, neutralizing the liquid, purifying with bentonite, precipitating the enzyme with methyl alcohol, dissolving in water and fractionating with methyl alcohol at low temperatures. By this procedure preparations were obtained showing about 80 times higher potency than the original pancreas. The preparations thus obtained were free from phosphatase, but contained ribonucleinase.

A method of determination of thymonucleodepolymerase, based on the liberation of acid-soluble phosphorus, was described. A "P-unit" of thymonucleodepolymerase was defined as an amount of enzyme which, when incubated with 25 mg. of thymonucleic acid in 0.2 *M* borate buffer pH 7.1, made 0.025 *M* in respect to  $\text{MgSO}_4$  (total volume 8 cc.), will liberate 100  $\gamma$  of acid-soluble phosphorus when incubated 60 minutes at 37°C.

By this method the influence of the concentration of the substrate and the enzyme on activity of thymonucleodepolymerase, as well as the time-activity relationship, was investigated, and the results were presented.

Previously shown by the viscosity method, the accelerating effect of Mg ions was confirmed by the acid-soluble phosphorus method. Inhibition of thymonucleodepolymerase by high concentrations of NaCl was shown by both methods. From this evidence the conclusion was reached that one enzyme (provisionally called thymonucleodepolymerase) is responsible for the hydrolysis of thymonucleic acid, at least up to the stage in which the high viscosity value disappears, and all phosphorus is changed into an acid-soluble form.

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*Addendum.* After the present manuscript was completed the paper of McCarty (*J. Gen. Physiol.* **29**, 123 (1946)) appeared. The method of purification described by McCarty is superior to ours. His procedure yields a preparation having a potency about 22 times that of the original acid extract, while our best product (on the same basis) had a 10-fold potency, usually 7-fold.

# The Production of Riboflavin by *Mycobacterium smegmatis*

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## INTRODUCTION

In a preceding paper (1) the enzymatic formation of a yellow pigment produced by *Mycobacterium tuberculosis* var. *hominis* in presence of *p*-aminobenzoic acid (paba), was reported. Continuing these experiments, it was found that many other *Mycobacteria* are able to produce this pigment, such as *Myco. avium*, *Myco. tuberculosis* var. *bovis*, *Myco. berolinensis* and *Myco. smegmatis*. Besides paba-pigment, all these strains normally formed another water-soluble, fluorescent yellow pigment. It was found that there was a certain relationship between both pigments, namely, that those strains which produced the greatest amounts of the water-soluble pigment also produced the greatest amounts of paba-pigment.

The highest yields of both pigments were obtained with a strain of *Myco. smegmatis*. As it is well known that numerous strains of *Myco. tuberculosis* synthesize riboflavin (2, 3, 4, 5), we ascertained that riboflavin was present in normal cultures of *Myco. smegmatis*. An investigation was undertaken to study in detail the conditions under which this vitamin was formed. By this study it was hoped to gain further insight into the problem of the riboflavin synthesis by microorganisms, of which very little is known at present. For this purpose, *Mycobacteria* seemed especially suitable, as they are almost autotrophic, whereas all other microorganisms known as riboflavin producers, for instance, *Eremothecium ashbyii* (6, 7, 8), *Azotobacter vinelandii* (9), *Escherichia coli* (10), *Staphylococcus aureus* (11) and *Bacterium dysenteriae* (12) grow optimally only on more or less complex media containing various organic nitrogen sources.



## EXPERIMENTAL

Three different strains of *Myc. smegmatis* obtained from the American Type Culture Collection, Strains Nos. 101, 361 and 278, were examined. If not otherwise mentioned, Strain No. 101 was used in the following experiments. The organisms were cultured on Long's medium of the following composition: Asparagine 0.5%, ammonium citrate 0.5%, potassium phosphate (primary) 0.3%, sodium carbonate 0.25%, sodium chloride 0.2%, ferric chloride .005%, glycerol 5%, and magnesium sulfate 0.1%, pH adjusted to 7.0. The comparative experiments were carried out in 18 mm. test tubes, each containing 10 ml. of medium, or in 250 ml. Erlenmeyer flasks, each containing 30 ml. of liquid. In all experiments in which one or more of the constituents of the medium were varied, Long's medium of double strength was brought to normal strength by the addition of neutralized solutions of the various compounds under investigation. The size of the inoculum was uniformly 1% of the medium used and the inoculum contained, unless otherwise noted, as in the previous publication (1), about 50  $\gamma$  of wet bacilli for 10 ml. of culture medium.

The tubes were incubated at 37°C. for 8-10 days, during which time about 10% of the culture medium evaporated. This amount was replaced by adding 1 ml. of distilled water in cases in which the filtrate was used before destroying the bacteria, or 1 ml. of 10% acetic acid solution in cases in which an estimation was planned after autoclaving the material. Preliminary experiments revealed that the bacteria retained only small amounts of riboflavin. However, a complete extraction and preservation of the vitamin could be assured when the tubes were autoclaved after acidification. After autoclaving, the contents of the tubes were individually filtered through weighed filter paper and the filtrate used for photofluorometric or biological determinations. The bacilli on the filter paper were thoroughly washed with distilled water, dried at 100°C. and weighed.

## EXPERIMENTAL IDENTIFICATION OF THE PIGMENT AS RIBOFLAVIN

The color of the solution containing the water-soluble pigment produced by *Myc. smegmatis* is bright yellow, very similar to the color of a solution of pure riboflavin; however, after prolonged incubation it becomes somewhat darker. There is a great difference when the two solutions are examined under Wood's light: Long's medium containing the *Myc. smegmatis* pigment gives a bluish-white fluorescence, whereas a pure riboflavin solution or a culture of *Eremothecium ashbyi*\* in Czapek-Dox' medium yields a yellow-green fluorescence. We, therefore, investigated by physico-chemical and biological methods as described below the identity of the fluorescent pigment.

## (A) Microbiological Determination

The microbiological determinations of the riboflavin content of filtrates carried out according to the method of Snell and Strong (13) indicated that after 10 days incubation, from 10 to 16  $\gamma$  riboflavin/ml. were usually present.

## (B) Photofluorometric Determination.

Numerous photofluorometric determinations of filtrates obtained under various conditions of growth gave values which agreed with the microbiological determinations, as seen in Table I.

\* We are indebted to J. W. Foster of Mores & Company for supplying this culture.

TABLE I  
*Comparison Between Microbiologic and Photofluorometric Assay*

Sample	Microbiological assay vitamin B <sub>2</sub> $\gamma$ /ml.	Fluorometric assay vitamin B <sub>2</sub> $\gamma$ /ml.
1	10.50	10.45
2	9.30	9.88
3	20.00	17.28
4	38.20	30.40
5	4.25	5.70
6	5.00	10.45
7	18.35	15.77
8	31.30	31.30

TABLE II  
*Riboflavin Determination by Rat Assay*

Group	No. of rats	Diet	Average daily weight increase
A	5	B <sub>2</sub> -free basal diet	0.6 g.
B	5	Basal diet + 10 $\gamma$ pure B <sub>2</sub> daily	1.86 g.
C	10	Basal diet + 10 $\gamma$ (by assay) unknown substance daily	1.80 g.

(C) *Rat Assay for Riboflavin (Bacon's Method (14)).*

For this experiment a culture filtrate which contained, according to fluorometric determination, 10  $\gamma$  of riboflavin/ml., was concentrated to one-twentieth of the original volume. The results are recorded in Table II. This experiment shows that 10  $\gamma$  of the investigated pigment elicits a daily weight increase almost equal to that obtained when an identical amount of pure crystalline riboflavin is used.

Further identification of the pigment as riboflavin was obtained by irradiation of the *Myco. smegmatis* filtrate in alkaline solution according to P. Karrer *et al.* (15). A chloroform-soluble, yellow-green fluorescent material was obtained, as was the case with a solution of pure riboflavin similarly treated. Irradiation of acid solutions yielded, in both cases, a bright blue-white fluorescence in the chloroform layer, typical of lunnichrome which is formed under these conditions.

It was found by R. Kuhn *et al.* (16) that riboflavin in fresh milk and in other natural sources is 90% dialyzable. The culture filtrate of *Myco. smegmatis* was dialyzed at 0°C. against distilled water; 93% of the pigment dialyzed in 72 hours.

It was concluded from this group of experiments that the yellow pigment produced in the culture medium by *Myco. smegmatis* is riboflavin and that the amount produced can be determined with sufficient accuracy by fluorometric methods. The bluish-white fluorescence of our filtrates which was mentioned previously was found

to be due to the presence of glycerol in Long's medium. Indeed, *Myco. smegmatis* grown in glucose medium gave a filtrate with bright yellow-green fluorescence.

## INFLUENCE OF ENVIRONMENTAL FACTORS ON VITAMIN B<sub>2</sub> PRODUCTION

### *Rate of Flavin Production*

The yield of vitamin B<sub>2</sub> depends largely upon the rapidity of growth, the relationship between surface and depth of the medium and the amount of the inoculum. As in similar fermentations, the greatest amounts were obtained in flasks and shallow layers. Under the conditions of our experiments, using test tubes with 10 ml. of medium, the maximum was generally reached after 8-12 days of growth as shown in Table III. In this experiment, because of a relatively small inoculum, growth was delayed.

TABLE III  
*Influence of Time on Growth and Riboflavin Production*

1	2	3	4	5
Days after inoculation	Dry weight of bacteria	Vitamin B <sub>2</sub>	Mg. vitamin B <sub>2</sub> /g. dry bacteria	Remarks
	mg./10 ml.	γ/ml.		
5	1	0	0	Inoculum: 10 γ/10 ml.
6	13.5	0.9	0.69	
7	38	6	1.58	
8	52	11.1	2.14	
9	52	11.0	2.12	
10	71	12.5	1.76	
11	82	15	1.82	
12	88	15.5	1.76	
13	84	14	1.67	

In all experiments in which the growth-rate and flavin-synthesis were recorded daily, the absolute values for riboflavin (column 3) and the values relative to the growth of the bacteria (column 4), do not parallel each other. The maximum of the relative values in column 4 of Table III, for instance, was reached on the eighth day, whereas the maximum of the absolute values in column 3 coincided with the twelfth day of growth.

The maximum level of riboflavin usually remained unchanged for several days. After 12-15 days of incubation the flavin content regularly drops more or less rapidly. Since the weight of the pellicle increases for several days, although the vitamin content of the medium decreases, the ratio between the amount of riboflavin and weight of bacteria then decreases rapidly.

The yields varied in different experiments. In the experiment described in Table III. 15.5 γ/ml. were obtained; in others, 30 γ/ml. or more.

In several experiments the effects of aerating or shaking cultures instead of the usual stationary cultures were studied. All our strains, however, had a strong tendency to form mats and adhere to the sides of the vessels, so that no more riboflavin was produced in this way.

#### *Influence of the Nitrogen Source*

Nitrogen is supplied, in Long's medium, as an ammonium salt and as asparagine. The influence of increased amounts of organic nitrogen and the influence of different nitrogen sources were studied first. Next the nitrogen was supplied either as inorganic nitrogen alone, in the form of ammonia, or as organic nitrogen in the form of asparagine, glycine or other amino acids and urea. These experiments led to the conclusion that the form in which nitrogen is present influences both the rate of growth and the yield of riboflavin.

An increase of asparagine strongly increased growth but caused a decrease in the amounts of riboflavin formed (Table IV).

TABLE IV  
*Influence of Asparagine on Growth and Vitamin B<sub>2</sub> Production*

Per cent asparagine in medium	Dry weight of bacteria	Vitamin B <sub>2</sub>	Mg. Vitamin B <sub>2</sub> /g. dry bacteria
	mg./10 ml.	γ/ml	
0	43	18	4.2
0.05	50	15.7	3.15
0.5	69	11.5	1.67
1.0	84	5.2	0.62
2.0	103	5.8	0.56

*Substitution of other amino acids for asparagine* leads to varied results. *Glycine* provided less growth and less riboflavin than asparagine (glycine inhibited growth in a concentration of 2%). Replacement of asparagine by *L-aspartic acid*, *DL-methionine*, *DL-leucine*, *DL-glutamic acid* or *L-arginine* did not substantially change either the growth or flavin synthesis. As with asparagine, the relative vitamin B<sub>2</sub> production decreased when growth increased. This is a general observation with a large number of riboflavin producers, such as *Aspergillus niger* (17, 18), *Candida guilliermondia* (19), and others. It is supposed that under the influence of growth-inhibiting factors, the cell walls become permeable to riboflavin which diffuses into the medium. To replace the essential vitamin lost by the cell, hyperproduction of riboflavin must take place which, in turn, leads to an accumulation in the medium. However, decrease of growth apparently does not always coincide with an increase of riboflavin.

*Omission of any organic nitrogen source* increased the yield of riboflavin. In experiments in which nitrogen was present in form of ammonium citrate or ammonium sulfate, values as high as 57.5 γ/ml., or 8.6 mg./g. dry bacteria were obtained.

Ammonium nitrogen could be replaced by nitrate nitrogen with resulting good growth and flavin production. Thus, *Myco. smegmatis* grows in media containing NO<sub>3</sub><sup>-</sup> in the same way as does *Myco. lacticola* (20), and is able to reduce NO<sub>3</sub><sup>-</sup> to the components necessary for the introduction of nitrogen into the riboflavin molecule.

*Influence of the Carbon Source*

Carbon sources in Long's medium are ammonium citrate, glycerol and asparagine. Experiments in which one or more of these three compounds were omitted gave the following results: Asparagine alone, citrate alone, or both together are unable to induce growth, the organism developing only in presence of glycerol (or certain sugars). On the other hand, glycerol alone is apparently not sufficient for growth; if citrate or, to a lesser degree, asparagine is simultaneously present with glycerol, the bacilli develop more luxuriantly. As far as the flavin synthesis is concerned, a comparison of the various data of Table V seems to indicate that a certain interrelationship exists between the three carbon sources.

TABLE V

*Influence of the Carbon Source on Growth and Vitamin B<sub>2</sub> Production*

Glycerin	Citrate	Asparagine	Dry weight of bacteria	Vitamin B <sub>2</sub>	Mg. vitamin B <sub>2</sub> / g. dry bacteria
			mg./10 ml.	$\gamma$ /ml.	
present	present	present	85	10	1.3
—	present	present	0	0	—
present	—	present	40	7	1.6
present	present	—	67	33	8.3
—	—	present	0	0	—
—	present	—	0	0	—
present	—	—	27	7	2.8

*Substitution of Glycerol by Glucose*

Glucose can favorably replace glycerol for growth, but not for riboflavin production. While great amounts of riboflavin were formed when only glycerol and inorganic nitrogen were present, only traces of vitamin B<sub>2</sub> appeared with glucose unless asparagine was added. It seems, furthermore, that asparagine cannot be replaced by glycine when glucose is present instead of glycerol. In any event, the amounts of

TABLE VI

*Influence of Glycerin and Glucose on Growth and Vitamin B<sub>2</sub> Production*

Asparagine	Glycerin	Glucose	Dry weight of bacteria	Vitamin B <sub>2</sub>	Mg. vitamin B <sub>2</sub> / g. dry bacteria
per cent	per cent	per cent	mg./10 ml.	$\gamma$ /ml.	
0.5	5	0	55	11.6	2.4
0.5	10	0	28	43.0	15.4
0.5	0	5	98	3.9	0.4
0.5	0	10	37	4.0	1.08
0	5	0	43	17.9	1.1
0	0	5	100	0.2	—

riboflavin formed in the presence of glucose were always very low, as seen in Table VI. No explanation can be offered for this behavior.

Glycerol is not the only carbon source which can be used in the production of vitamin B<sub>2</sub>; in many cases certain sugars are even superior.

A comparison of the influence of twelve different sugars is given in Table VII. Five sugars did not produce more than traces of vitamin B<sub>2</sub> (galactose, saccharose, lactose, trehalose and maltose). Levulose was the best carbon source for growth and riboflavin production. Xylose gave relatively the highest yields, although it led to only very limited growth. Two other strains of *Myc. smegmatis* (Nos. 278 and 361) gave similar results. An increase of glucose concentration from 5 to 10% did not increase the riboflavin production although it decreased growth. On the contrary, increase of the glycerol concentration from 5 to 10% yielded the highest vitamin concentration observed in our experiments, namely, 123  $\gamma$ /ml., or 36 mg./g. dry bacteria.

TABLE VII

*Influence of Various Sugars on Growth and Vitamin B<sub>2</sub> Production*

Sugar	Dry weight of bacteria	Vitamin B <sub>2</sub>	Mg. vitamin B <sub>2</sub> /g. dry bacteria
	mg./10 ml.	$\gamma$ /ml.	
Arabinose	87	6.70	0.77
Mannitose	86	1.61	0.19
Levulose	91	11.19	1.23
Glucose	69	1.61	0.23
Galactose	17	0.57	0.34
Inositol	83	2.01	0.24
Sorbitol	72	2.21	0.31
Saccharose	14	0.07	0.05
Lactose	16	0.17	0.11
Trehalose	32	0.07	0.02
Maltose	33	0.44	0.13
Xylose	10	2.72	2.72

As seen above, citrate influences growth very strongly if another carbon source, such as glycerol or a sugar, is present. Citrate was replaced by the following acids (as the ammonium or sodium salt(s)): formic, acetic, fumaric, maleic, pyruvic, lactic, tartaric and succinic. Formic acid was definitely toxic in concentrations of 0.5% or more; acetic acid in concentrations of 1% and above; all others were non-toxic at concentrations of 2-5%. If used in amounts ranging from 0.1% to the highest non-toxic concentration, none of these acids was as effective as citrate in growth and riboflavin promotion. However, they uniformly increased growth from two to three times, as compared with controls in which none of the acids were present. They differed, however, in their influence on riboflavin production. A definite improvement over controls upon the yield of riboflavin was seen with the following acids: maleic, fumaric and, especially, tartaric acid. It might be possible that the presence of citrate chiefly influences the state of ionization of magnesium necessary for growth and flavin-production.

*Influence of the Inorganic Ions Present in Long's Medium**Cl<sup>-</sup>, PO<sub>4</sub><sup>-</sup>, K<sup>+</sup>, Mg<sup>++</sup>, SO<sub>4</sub><sup>-</sup>, trace elements, Hg.*

Cl does not contribute to growth or riboflavin production; PO<sub>4</sub><sup>-</sup> and K<sup>+</sup> are essential for growth. With increasing amounts of K<sup>+</sup>, growth gradually increases and vitamin B<sub>2</sub> production parallels this increase (Table VIII). The experiment shown in

TABLE VIII

*Influence of Potassium on Growth and Vitamin B<sub>2</sub> Production*

Mg. % KCl	Dry weight of bacteria	Vitamin B <sub>2</sub>	Mg. vitamin B <sub>2</sub> /g. dry bacteria
	mg./10 ml.	γ/ml.	
0	8	0.2	0.25
0.5	15	2	1.33
1	10	3	3.0
2.5	22	5.25	2.38
5	38	5.25	1.38
10	44	8.5	1.93
25	57	15.5	2.72
50	63	15.5	2.46
100	60	15	2.50
500	60	20.5	3.43
1000	60	18.5	2.80
2000	52	14.5	2.78
2500	62	13.5	2.17

this table was made with a potassium-free medium in which the potassium salt was replaced by the corresponding sodium salt, and to which varying amounts of KCl were added. The optimum growth was observed with 50 mg.% of KCl; the optimum riboflavin content with 500 mg.% of KCl. Higher concentrations of KCl adversely affected the riboflavin synthesis more than they did growth.

No influence of sodium upon growth or vitamin B<sub>2</sub> production was observed. However, in one experiment, the omission of sodium resulted in an especially high vitamin B<sub>2</sub> production.

Mg<sup>++</sup> ions and SO<sub>4</sub><sup>-</sup> ions are both indispensable. Magnesium, which has a great influence upon the development of the paba-pigment, also influences the flavin synthesis strongly. Magnesium is present in normal Long's medium as MgSO<sub>4</sub>. This substance could not be replaced by MgCl<sub>2</sub> unless Na<sub>2</sub>SO<sub>4</sub> was added, and Na<sub>2</sub>SO<sub>4</sub> alone did not permit growth unless MgCl<sub>2</sub> was added. It is difficult to determine the role of each one of these ions, although it seemed that the sulfate ion is more essential for growth than for the production of riboflavin. Magnesium definitely influences growth, but it also appears to be involved in B<sub>2</sub> production (Table IX). It enhances riboflavin production in amounts which hinder growth, but this might well be explained as an indirect influence, the increased vitamin output being the consequence of diminished growth as such.

TABLE IX  
Influence of  $MgSO_4$  on Growth and Vitamin  $B_2$  Production

Mg. % $MgSO_4 \cdot 7H_2O$	Dry weight of bacteria	Vitamin $B_2$	Mg. vitamin $B_2$ /g. dry bacteria
	mg./10 ml.	$\gamma$ /ml.	
0	8	0.2	0.25
10	42	5.2	1.23
50	46	14.0	3.05
100	59	13.9	2.36
500	52	24.9	4.78
1000	47	29.7	6.35
2000	37	14.6	3.95

The highest amounts of riboflavin are formed when concentrations of  $MgSO_4$  of 0.5–1% are present, <sup>†</sup> with growth optimal at 0.1%.

Iron had no definite effect upon the riboflavin production, although in some experiments the yields were somewhat smaller when iron was lacking. This was apparently due to the influence of iron upon growth, which was normal only when 1  $\gamma$  of iron/ml. was available.

Cu, Zn, Cr (as  $K_2Cr_2O_7$ ) or Hg (as  $HgCl_2$ ) had no influence.

Mn (as  $MnCl_2$ ) in concentrations of 0.5 to 5  $\gamma$ /ml. had a definite stimulatory effect on growth and riboflavin.

The ineffectiveness of  $HgCl_2$  is worth mentioning as it is claimed to have a definite effect on the vitamin  $B_2$  production by *Aspergillus niger* (17, 18).

HCN, in concentrations which did not impair growth, did not influence the flavin synthesis.

TABLE X  
Influence of pH on Growth and Vitamin  $B_2$  Production

pH	Dry weight of bacteria	Vitamin $B_2$	Mg. vitamin $B_2$ /g. dry bacteria
	mg./10 ml.	$\gamma$ /ml.	
4.3	—	—	—
4.7	1	0.27	—
5.2	78	16.9	2.16
6.0	66	32	4.85
6.6	80	27.5	3.44
7.0	79	6.25	0.8
7.5	79	8.5	1.08
8.2	82	9.1	1.1

\* An insoluble red pigment appeared in the pellicle, showing a red fluorescence when high concentrations of  $MgSO_4$ ,  $FeCl_3$  or glucose (10–20%) were employed.



### *Influence of pH*

*Myco. smegmatis* grows over a wide pH range. Slight growth occurs at pH 4.7, normal growth from pH 5.2 to pH 8.2. The production of vitamin B<sub>2</sub> starts with the first visible growth and is especially heavy at pH 6, but is less at pH 7 and above (Table X.)

Since riboflavin is unstable on the alkaline side, it might be possible that low values above pH 7 are due to a rapid destruction of the vitamin rather than to low production.

### DISCUSSION

Few attempts have been made to investigate the biosynthesis of riboflavin. The most complete studies were performed with the yeast *Candida guilliermondia*, by Burkholder (19), and the mold *Eremothecium ashbyii*, by Schopfer (8). Examination of numerous environmental factors showed that for each organism, individual conditions prevailed. Since both organisms are more or less related botanically, it seemed useful to investigate a non-related organism with high riboflavin-producing activity in order to obtain, through comparison, a better insight into the mechanism involved in the biosynthesis of this vitamin. The use of *Myco. smegmatis* for such a study seemed especially interesting for several reasons. First, *Myco. smegmatis* is considered a bacterium, although certain relationships exist between *Mycobacterium* and molds. Second, it is certainly one of the most prolific producers of this vitamin among the *Mycobacteria*. *Myco. tuberculosis* var. *hominis*, for instance, produces, according to Rohner and Roulet (3), about 0.15–0.2 mg. *Myco. smegmatis* regularly yielded 2–5 mg./g. up to a value as high as 36 mg./g. dry bacilli under special conditions. Third, *Myco. smegmatis* is an organism which needs, for full growth and vitamin production, no medium-constituents other than PO<sub>4</sub><sup>-</sup>, SO<sub>4</sub><sup>-</sup>, Mg<sup>++</sup>, K<sup>+</sup> and NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> ions and a simple carbon source, for example, glycerol or a sugar.

In comparing the results of the studies on the biosynthesis of riboflavin with those which had been previously obtained in our study of the formation of the paba pigment, some conclusions may be drawn regarding the influence of environmental factors on either growth or riboflavin production.

The presence of an organic nitrogen source is of paramount importance for the flavin synthesis by *Eremothecium ashbyii*. Peptones and certain natural extracts serve as a nitrogen, or as a vitamin, source. Moreover, the addition of asparagine or glycine influences growth slightly, as a rule, but strongly influences the flavin synthesis. A syn-

ergism also exists between peptones and vitamins contained in the medium. Unlike this mold, the yeast *Candida guilliermondia* is capable of forming a certain amount of riboflavin in absence of organic nitrogen (10). However, the addition of small amounts of amino acids enhances the vitamin B<sub>2</sub> production. This enhancement depends upon the chemical nature of the various amino acids. The presence of *dl*-glutamic acid doubled the yield of riboflavin over that obtained in the absence of organic nitrogen; similarly, *dl*-aspartic acid tripled the yield, and *dl*-methionine quintupled the riboflavin content. On the other hand, an increase of asparagine from 0.1 to 0.8% tripled the amounts of vitamin formed (10).

According to these results, the flavin synthesis of *Candida* and *Eremothecium* seems to be stimulated by amino acids in a similar manner. The case of *Myco. smegmatis* is quite different, for the addition of small amounts of amino acids does not influence its riboflavin production, while additional amounts markedly decreased flavin formation by this organism. Such concentrations, on the other hand, stimulate the vitamin synthesis of *Eremothecium* and *Candida*. Growth increased proportionally with the amount of amino acids added. The most interesting feature with *Myco. smegmatis* is that maximal amounts of riboflavin were obtained when only inorganic nitrogen was present.

This dissimilar behavior of the three organisms points either to different mechanisms in the riboflavin synthesis or to the fact that organic nitrogen is used chiefly for growth, its positive influence upon vitamin production in the case of the mold and the yeast being only a secondary effect due to the improved growth. We favor the second alternative because we are inclined to assume, at least as a working hypothesis, that the various organisms employ similar procedures in elaborating a specific compound such as riboflavin.

Great differences also exist in the utilization of different sugars by the yeast, mold and *Mycobacterium*. In the case of *Eremothecium ashbyii*, maltose, saccharose, glucose and levulose are particularly favorable for flavin production, whereas lactose and galactose act to a much lesser degree. *Candida guilliermondia* gives the highest amounts with mannose, saccharose, glucose and levulose. *Myco. smegmatis*, on the contrary, gives very small yields with saccharose, glucose and mannose, but very good yields with arabinose and levulose. Levulose is the only sugar which gives high yields with all three organisms (Table XI).

TABLE XI

*Influence of Various Sugars upon the Flavin Synthesis by Different Organisms*

Sugar	Growth			Riboflavin		
	<i>Myco smegmatis</i>	<i>Erem ashbyi</i>	<i>Candida guilliermondia</i>	<i>Myco smegmatis</i>	<i>Erem ashbyi</i>	<i>Candida guilliermondia</i>
Arabinose	++++	++		++++	++	++
Mannitose	++++	++	+++	++	(+)	++
Levulose	++++	+++	++++	+++++	+++	+++++
Glucose	+++	+++	+++	+	+++++	+++++
Galactose	+	++	++++	(+)	(+)	(+)
Inositol	++++	++		++	+	
Sorbitol	+++	++++	++++	++	(+)	++
Saccharose	+	++++	++++		+++++	+++++
Lactose	(+)	++	++	(+)	+	+
Trehalose	++	+++++			++	
Maltose	++	++++	++++	(+)	++	(+)
Xylose	(+)	+		++	+++	
Glycerine	+++		++	++		++

(+) Traces of growth.

+ Definite, but small, growth.

++ to ++++ Good to maximum growth.

This comparison suggests that two factors determine the role which a specific sugar plays in the biosynthesis of riboflavin. The first is its intrinsic ability to serve as building material for the molecule and, in the case of di- or polysaccharides, the ability of the riboflavin-producing organism to split these sugars to monosaccharides. Saccharose, for instance, is easily hydrolyzed by yeast and, apparently, also by *Eremothecium*, since both organisms thrive with this sugar as a carbon source and produce great amounts of riboflavin, as they do with glucose. In the presence of saccharose, *Myco. smegmatis* grows slightly and does not form any riboflavin. We conclude that this organism, like most bacteria, does not produce invertase. It would certainly be interesting to extend this investigation to other sugars and to acquire further knowledge on the formation of *d*-ribose.

There is no proof that any one of the inorganic components of the culture medium for *Myco. smegmatis* is especially involved in the

flavin synthesis. Certain ions, as for instance,  $\text{PO}_4^{=}$ ,  $\text{SO}_4^{=}$ ,  $\text{K}^+$  or  $\text{Mg}^{++}$ , are highly important for growth and vitamin production, and, for the time being, it is impossible to determine whether their influence upon the latter is primary or only secondary. The results with various concentrations of iron and the effect of HCN have a theoretical importance. Iron seems to be necessary only for growth, but the riboflavin synthesis shows only very slight dependence, if any at all, upon the concentration of iron in the medium. In the presence of HCN, the flavin synthesis decreases proportionately to the decrease of growth. The same observation was made by Burkholder in the case of *Candida guilliermondia*, where HCN produced a transient inhibition of growth and  $\text{B}_2$  formation but, after a certain time, the yeast began to grow and produce good yields of riboflavin. In other instances HCN appreciably increases the production of riboflavin by various microorganisms.

The lack of importance of iron and the ineffectiveness of HCN would indicate that the production of riboflavin by *Myco. smegmatis* is independent of a cytochrome system and that probably no other iron-containing enzyme system is involved.

Iron was found to be important for the elaboration of the paba pigment, since HCN strongly influenced its formation. It was concluded from these results that the paba-oxidase was possibly an iron-containing enzyme. In spite of the apparent relationship between the formation of the paba pigment and of riboflavin which has been discussed elsewhere in this paper, the paba-oxidase does not seem to be involved in the flavin synthesis. The parallelism between both processes might be of a quantitative nature inasmuch as those organisms which contain greater amounts of paba-oxidase are generally richer in other enzymes, such as those which lead to riboflavin production.

It is certain that the nitrogen in the riboflavin molecule is, or can be taken from, inorganic nitrogen. This is certainly true for *Myco. smegmatis* and it can be true for the two other organisms, where this process might be masked by the presence of organic nitrogen necessary for growth. Urea is not a direct intermediate in the nitrogen metabolism, since it is not used either by *Myco. smegmatis* or by *Candida guilliermondia* (19). On the contrary, it is toxic. There is a possibility that the arginine-ornithine cycle is involved. Experiments are under way to elucidate this point.

There is no doubt that the investigation of this whole problem is especially complicated by the strong interrelationship of growth and

flavin-synthesis. It is difficult to discern, with the commonly used methods, which building material is specifically used for the production of riboflavin and which serves for the somatic development of the microorganisms. Many questions might be solved in studying the cell-free riboflavin-synthesizing enzyme system isolated from one of the vitamin producers and also by using building material with tagged nitrogen and/or carbon atoms.

### SUMMARY

*Myc. smegmatis* under certain conditions produced significant amounts of a water-soluble yellow pigment, identified as riboflavin. Various factors, particularly the nitrogen and carbon sources, were studied to determine their role in this biosynthesis of riboflavin. It was found that the highest yields of flavin were obtained in the absence of organic nitrogen. The utilization of carbohydrates followed no definite pattern. A comparison of the factors influencing this synthesis and those involved in the synthesis of riboflavin by *Candida guilliermondia* and by *Eremothecium ashbyii* indicated that organic nitrogen is probably used for the growth of the organism and that the utilization of carbohydrates depends upon the ability of organisms to split the sugars to monosaccharides and whether the sugar can serve as building material for the molecule.

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Studies in Histochemistry  
XVII. Localization of Phosphatases in the  
Wheat Grain and in the Epicotyl and  
Roots of the Germinated Grain

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INTRODUCTION

The histochemical technique for the localization of phosphatases, which was described in the previous communication (1), was applied in this investigation to the wheat grain and the epicotyl and roots of the germinated grain. With the methods available, one must very often, as in the present case, sacrifice quantitation in histochemical investigations for fineness of localization, or *vice versa*. Hence, it would be most desirable to utilize the quantitative chemical methods, which have been developed for studies on microtome sections of tissue, in addition to the methods designed to reveal the finer chemical morphology. The present communication is confined exclusively to the latter and constitutes no more than a preliminary step in the investigation of this phase.

METHODS

Paraffin sections of wheat grains, epicotyls and roots, and frozen sections of grains were prepared just as described in the preceding publication (1). A pure variety of Ceres wheat from the Montana 1945 crop was used exclusively. The roots and epicotyls of the sprouted grain were used before they attained a length of 5 and 3 mm., respectively. However, before the sections were used for the demonstration of enzyme activity they were placed in distilled water for 24 hours at room temperature, which was found to remove all free phosphate and other ions capable of giving a positive test. Separate tests showed that this treatment did not cause a significant loss in the

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enzyme activity in the sections. Gomori (2) found that sections may be treated with citrate buffer, pH 3.5, to remove preformed calcium precipitates without damaging either acid phosphatase or lipase.

The demonstration of enzyme activity with the substrates sodium glycerophosphate, thiamine pyrophosphate and adenosine-triphosphate (ATP) was carried out according to the directions previously given (1). The glucose-1-phosphate substrate was prepared by substituting this compound for an equal weight of thiamine pyrophosphate and making it up in the same fashion as the latter substrate. The solution was centrifuged before use to remove insoluble material.

A separate set of control sections which were not previously treated with water were placed in substrate-free media, *i.e.*, the acetate buffer solution containing only lead nitrate. These sections served to visualize free phosphate, primarily. Carbonate and chloride are not precipitated under the conditions employed.

## DISCUSSION

### *The Reaction in Control Sections*

The method applied to the control sections will, of course, visualize any insoluble lead compounds that might be formed by the histochemi-



FIG. 1

Aleurone cells in a control section of a wheat grain showing a positive reaction (chiefly for free phosphate) in the cytoplasm. The outer layers of the grain, above the aleurone region, are completely negative. (325  $\times$ )

cal reaction. However, as already indicated, the positive reaction is due chiefly to free phosphate. Since control sections which had been left in water for 24 hours gave a completely negative reaction, there was no insoluble material present in the sections that could be stained.

From Fig. 1, it is clear that the cytoplasm of the aleurone cells gives a very strong positive reaction. In this figure only three cells may be seen to have been cut in such a manner as to reveal their nuclei. The

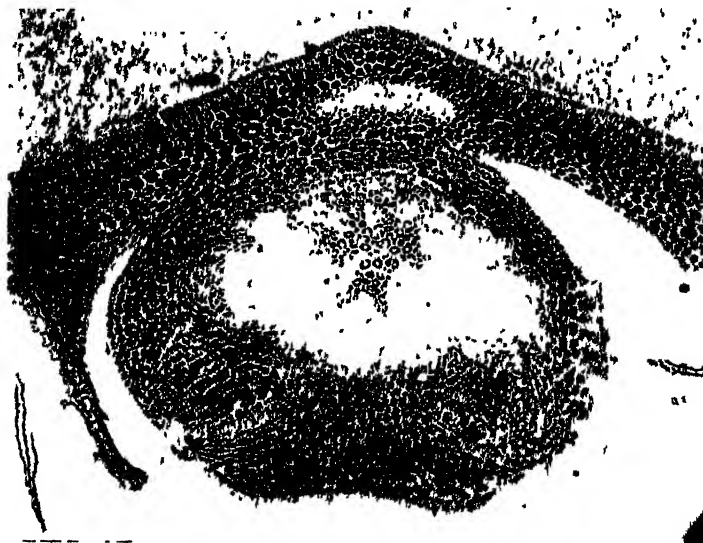


FIG. 2

A control section of wheat embryo showing a strongly positive reaction (chiefly for free phosphate) in the cytoplasm of the scutellum cells and a much weaker cytoplasmic reaction in some of the other embryo cells. Cell nuclei in all areas and the central vascular bundle in the scutellum are essentially negative, as are certain regions in the rudimentary leaves of the embryo. (60  $\times$ )

cytoplasm is filled with so-called "aleurone grains" and some of these may be seen on the surface of the nuclei. The nuclei themselves do not appear to react positively. The aleurone cell walls and the hyaline material between the cells gave a negative reaction as did all of the outer layers of the grain. The dark band above the aleurone cells is due to the natural brown pigment contained in this structure, the testa layer, and it does not constitute an even mildly positive reaction. At scattered

points in the endosperm some blackening was observed in the sections but this effect was most likely due to artifacts. Certainly, with the present method, no structure in the endosperm could be said to show free phosphate with any degree of consistency.

The regions giving a positive reaction in the scutellum and embryo are shown in Fig. 2. The strongest reaction is found in the cytoplasm of the cells in the scutellum. The cytoplasm gives a much weaker reac-



FIG. 3

Aleurone cell region in a section of a wheat grain showing a positive reaction for phosphatase (ATP substrate) in the cytoplasmic "aleurone grains" and in the cell walls and intercellular material surrounding the cells. The outer layers of the grain above the aleurone region are completely negative. (325  $\times$ )

tion, or none at all, in the rest of the embryo, and the cell nuclei are uniformly negative throughout the entire germ. The central vascular bundle in the scutellum is essentially negative.

No reaction could be demonstrated in any of the sections of the epicotyl or root of the germinated grain. Only paraffin sections of these tissues could be used, and it is possible, and even probable, that free phosphate exists in these structures, but it is lost during the treatment of the material with the various solvents employed in the

histological technique. This difficulty might be circumvented by the use of the Packer-Scott (3) freezing-drying apparatus which enables the preparation of paraffin sections without the use of fixing and dehydrating liquids



FIG 4

A section of wheat embryo showing a positive phosphatase reaction (glucose-1-phosphate substrate) particularly in the scutellum, and in the caps of the roots, right and left upper center (38 X)

#### *The Distribution of Phosphatases*

The specificity of the enzymes that liberate phosphate from the four substrates employed in this study has not been entirely elucidated. As Moog and Steinbach (4) have pointed out, there are instances where it is questionable whether the present method for the demonstration of the hydrolysis of ATP will visualize ATPase or phospho-

monoesterase. However, it has been emphasized (5) that the histochemical method can differentiate between the two enzymes when they have different cellular distributions, or when only one is predominantly present. The same considerations might apply to the enzymatic hydrolysis of the other substrates employed. In certain instances in this investigation it will be seen that there is an actual difference in the histological disposition of the enzymes that act on the respective sub-

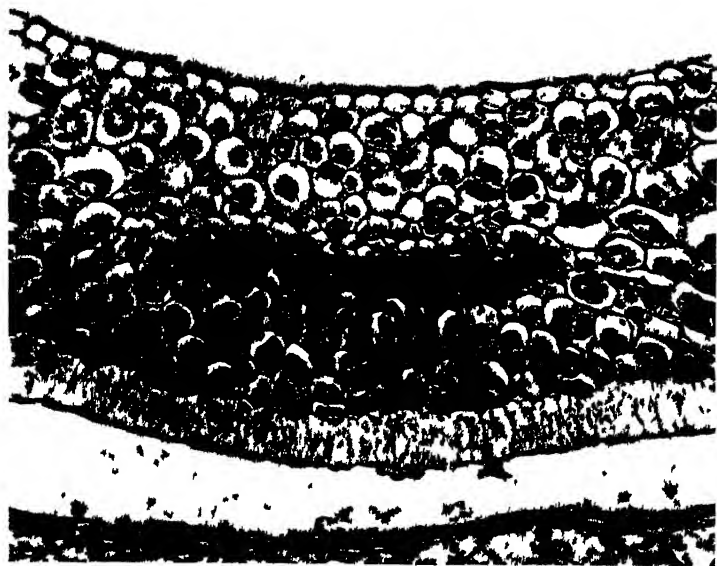


FIG. 5

A section of the scutellum showing a positive phosphatase reaction (glucose-1-phosphate substrate) in the central bundle, cell walls and cytoplasm. The columnar epithelium on the lower edge of the scutellum is negative in this case, but in other sections cytoplasmic granules in these cells were positive. (185  $\times$ )

strates, and in other cases this difference does not exist. It is perhaps preferable not to specify the existence of particular narrowly defined phosphatases in the material studied, and rather only to describe the localizations observed when the different substrates were employed.

It should be pointed out that two possibilities exist when glucose-1-phosphate is used. Either phosphatase action might occur to split off the phosphate, or phosphorylase action might be present to synthesize

starch from the substrate with the liberation of phosphate. Both enzymes would give a positive reaction in the method used, and the presence of both enzymes is possible. Since it has been found that phosphorylase requires the presence of some starch to initiate its action, soluble starch was added to the glucose-1-phosphate medium,



FIG. 6

A section of wheat embryo showing a positive reaction for phosphatase (ATP substrate), most pronounced in the vascular bundles, and prominent in the scutellum and roots. (80  $\times$ )

but no intensification of the reaction was observed in the sections. This may have been due to the presence of wheat starch already in the sections. The proof of whether the reaction obtained with glucose-1-phosphate results from phosphatase, phosphorylase, or both, will depend on further investigation.

*Phosphatase in the Wheat Grain.* No differences were found in the

phosphatase reaction obtained with any of the four substrates in the aleurone cell region of the grain. The photomicrograph of a representative section is shown in Fig. 3. The outer layers of the grain gave no reaction, but the cell walls and the hyaline material between the aleurone cells were strongly positive. It will be recalled that these regions were negative in the control sections, Fig. 1. The cytoplasmic "aleurone grains" showed enzyme activity but the nuclei of the cells

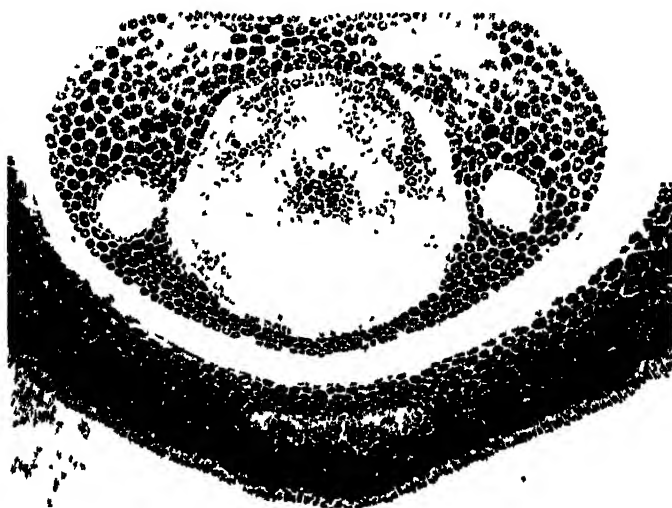


FIG. 7

A section of wheat embryo showing a positive phosphatase reaction (glycerophosphate substrate) in the scutellum and its columnar epithelium, and in certain other cells. The nuclei are negative, as are the cell walls and areas in the cross-section of the rudimentary leaves and bundles. (75  $\times$ )

were negative. No phosphatase activity could be ascribed with any certainty to particular structures in the endosperm. Some darkening was frequently observed in the outer endosperm layer adjacent to the aleurone cells. This was particularly noticeable in the cell walls, but may have been due to an adsorption reaction rather than true enzyme activity.

In the scutellum, which separates the bulk of the embryo from the endosperm, strongly positive reactions were found with all four sub-

strates, particularly in the cytoplasm of the cells. However, the nuclei were negative in all cases, *e.g.*, Fig. 5, except with thiamine pyrophosphate, in which instance they were positive. The central vascular bundle gave a very strongly positive reaction with glucose-1-phosphate (Figs 4, 5) and with ATP (Fig. 6) but was relatively negative with the other substrates (Figs. 7, 8). The columnar epithelium facing the endosperm showed a considerable lack of consistency. It was completely negative in some cases, but in others cytoplasm gave a positive

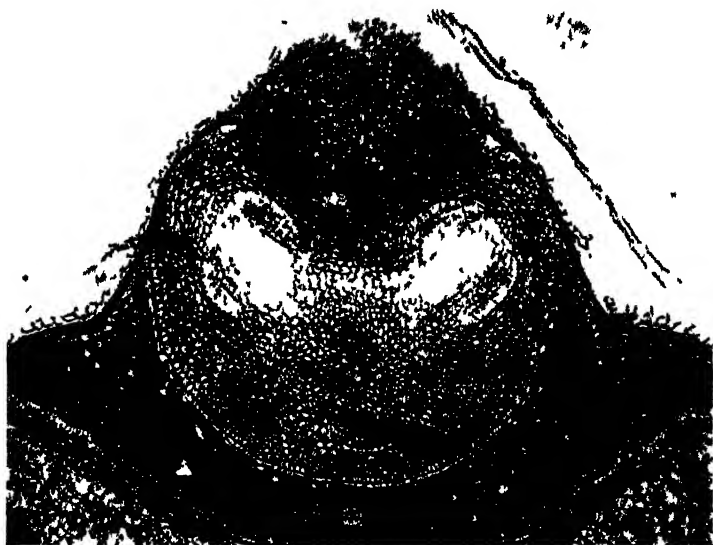


Fig. 8

A section of wheat embryo showing a positive phosphatase reaction (thiamine pyrophosphate substrate) particularly in the scutellum, root caps and cell walls. The central bundle in the scutellum gives a weaker reaction (60 X)

reaction with the same substrate. The nuclei were negative; however, in some sections positive nuclei and even more positive nucleoli were observed when glycerophosphate was used. These variations may be due to the changing physiological state of these cells which play a large role in the passage of nutriment from the endosperm to the embryo when germination is initiated. The mere soaking of the grains to soften them sufficiently for sectioning is accompanied by some of the early physiological changes.



In the cells of the radicle of the embryo the nuclei gave a positive reaction and the nucleoli an especially strong reaction with all of the substrates, *e.g.*, Fig. 6, except thiamine pyrophosphate. In this instance a diffuse weak reaction was given by the entire cell. It may be observed that the cell walls in the embryo react positively with thiamine pyrophosphate as the substrate (Fig. 8) and negatively with the other sub-



FIG. 9

A section of wheat epicotyl showing a reaction for phosphatase (thiamine pyrophosphate substrate), relatively diffuse in the inner leaves. (40 X)

strates. With all of the substrates the root caps showed a positive reaction as in Figs. 4 and 8.

*Phosphatase in the Epicotyl and Roots of Germinated Wheat.* With all the substrates the coleoptile, or outer leaf of the epicotyl, gave a stronger enzyme reaction than the inner leaves, and the tips of the coleoptile and leaves gave the most pronounced reaction. The cell

walls, particularly, were more positive in the coleoptile. A marked difference was observed in the epicotyl when thiamine pyrophosphate was used as substrate compared to the other compounds. In the low-power photomicrograph, Fig. 9, the more diffuse reaction in the inner



FIG 10

A section of wheat epicotyl showing a reaction for phosphatase (ATP substrate) nuclei in the inner leaves strongly positive (40  $\times$ )

leaves with thiamine pyrophosphate is apparent. This is due to the fact that both the cytoplasm and nuclei of these cells give reactions of similar intensities in contrast to the more strongly reacting nuclei in the other cases, *e.g.*, Fig. 10. A further difference is evident from examination of the oil-immersion photomicrographs of the same sections (Figs. 11, 12) which show that the nucleoli in the nuclei of the section which had been treated with thiamine pyrophosphate are negative

while the nucleoli in the other case are positive. The difference in the reaction in the cell walls of the coleoptile and inner leaves, mentioned above, is also apparent in the oil-immersion photomicrographs.

Figs. 13 and 14 demonstrate phosphatase reactions in the emerged roots. The results with different substrates were essentially the same, except for thiamine pyrophosphate. This substrate gave relatively stronger reactions in the cell walls, as it did in the embryo (Fig. 8)

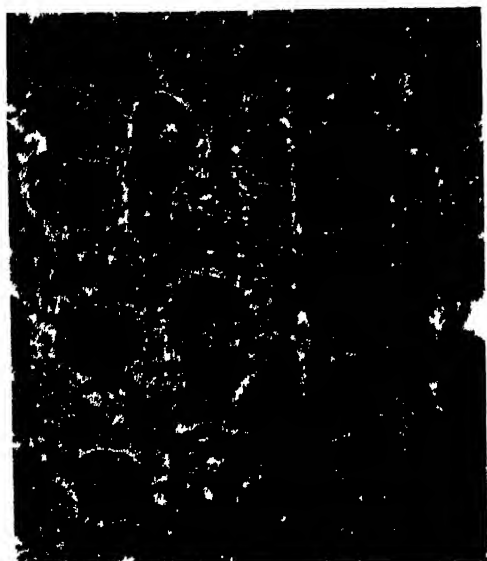


Fig. 11

Oil immersion photomicrograph of same section as in Fig. 9 showing a phosphatase reaction in the cell walls of the coleoptile on the right but none in the cell walls of the inner leaf on the left. The strongly positive nuclei contain nucleoli which are negative. (850  $\times$ )

and the nucleoli appeared to be negative, as they were in the epicotyl, Fig. 11. A parallelism may be drawn between the reaction given with the other three substrates in the emerged roots and in the embryonic roots before they emerge from the grain. In both stages of development the cells show positive nuclei with more strongly positive nucleoli.

A survey of the preceding descriptions reveals that, when thiamine pyrophosphate was employed as the substrate, differences from the

reaction given by the other substrates were obtained in the embryo, epicotyl and root. The central vascular bundle in the scutellum reacted positively with glucose-1-phosphate and ATP, but gave a weak reaction with the other two substrates. Positive reacting nuclei in the columnar epithelial cells of the scutellum were observed only with glycerophosphate, and then not in all instances; the reaction in these cells is highly variable, perhaps a reflection of the changing physiologi-



FIG. 12

Oil immersion photomicrograph of same section as in Fig. 10 showing a phosphatase reaction similar to that in Fig. 11 with the exception of the strongly positive nucleoli in the cell nuclei. (850  $\times$ )

cal state. With all of the substrates the aleurone cells showed strongly reacting cytoplasm and negative nuclei, and the cell walls and hyaline material between the cells was intensely positive in all cases.

#### ACKNOWLEDGMENT

The authors are grateful to Dr. A. O. Dahl of the Botany Department, University of Minnesota, for his helpful criticisms of the manuscript.

## SUMMARY

Descriptions have been given of histochemical localizations of certain phosphatases in the wheat grain, and in the epicotyl and roots of the germinated grain. Control experiments have indicated the distribution of free phosphate. Sodium glycerophosphate, adenosine



FIG. 13

A longitudinal section of the tip of a wheat root showing a phosphatase reaction (ATP substrate) most pronounced at the root cap and at the region behind the tip. (85  $\times$ )

triphosphate, glucose-1-phosphate and thiamine pyrophosphate were used as substrates for the enzyme studies. Both similarities and differences in the morphological distribution of the enzymes acting on these substrates were observed.

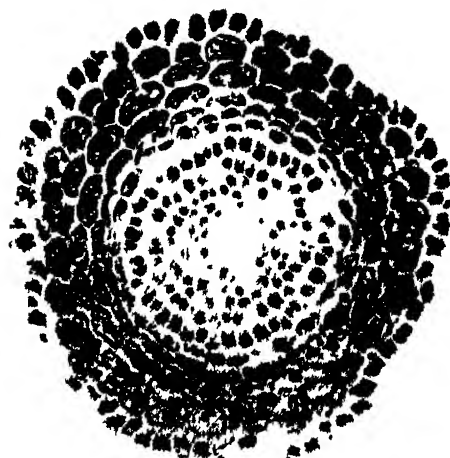


FIG. 14

A cross section of the tip of a wheat root showing a phosphatase reaction (glycero-phosphate substrate) stronger in the cortex and particularly positive in cell nuclei. (150 X)

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#### ADDENDUM

The recent method of Gomori (6) for the histochemical localization of lipase was applied to sections of the tissues used in the present study. Our results showed no reaction in any of the sections. This was confirmed by Dr. Gomori who kindly repeated the work in his own laboratory. Wheat is known to contain lipase, but the activity is usually of a low order. The restrictions of the method impose the use of a substrate which is water soluble and at the same time an ester of a fatty acid of high molecular weight, such as "Tween 40" (Atlas Powder Company). Conceivably, the wheat lipase has little or no action on this type of substrate.



# Studies in Histochemistry XVIII. Localization of Arginine in the Wheat Grain and in the Epicotyl and Roots of the Germinated Grain

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## INTRODUCTION

To gain information concerning the histological localization of amino acids and protein in the wheat grain and in the epicotyl and roots of the germinated grain, a survey of the available microchemical tests for these substances was made. It was found that the drastic nature of many of the procedures precluded their value for application to the microtome tissue sections which must be employed in the investigation.

Thus Romieu's reaction (1) for tryptophane could not be used because the tissue was disintegrated by the syrupy phosphoric acid used in the test. Millon's reaction for tyrosine, as adapted by Bensley and Gersh (2) to tissue sections, also proved impractical because of the strong acid in the reagent. The ninhydrin reaction for  $\alpha$ -amino acids, used histochemically by Berg (3), involves boiling the sections, a process disruptive to the tissues considered in this work.

Serra (4) recently described a method for the demonstration of arginine in tissue sections which we could adapt to our purposes, although this procedure, too, is rather drastic. The positive test depends on the development of a reddish-orange color by the action, in the cold, of an alkaline  $\alpha$ -naphthol-hypobromite reagent. The specificity of the reaction has been reported to be very great, negative results being given by guanidine, urea, ornithine, creatine, creatinine, asparagine, histidine and other amino acids. The only biological substances found to give a positive reaction besides arginine are the rare compounds, glycoeyamine, gelegine and agmatine.

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## METHODS

A pure variety of Ceres wheat from the Montana 1945 crop was used exclusively. The roots and epicotyls of the sprouted grain were used before they attained a length of 5 and 3 mm., respectively. As it is not possible to obtain satisfactory frozen sections of the roots and epicotyl, only paraffin sections 10  $\mu$  thick, of these tissues were employed. However, frozen grain sections, 15  $\mu$  thick, were used in preference to paraffin sections because the former gave stronger reactions.

*Arginine Reaction*

*Special Reagents.* Acetic alcohol-formalin fixative. Add a few drops of glacial acetic acid to each 10 ml. of a mixture of 2 vol. 96% ethyl alcohol and 1 vol. formalin.

1%  $\alpha$ -Naphthol in 96% alcohol. Store in a refrigerator; dilute 1:10 with 40% alcohol before use.

4% Sodium hydroxide.

2% Sodium hypobromite. With stirring and cooling, add 2 g., or approx. 0.7 ml., of bromine to 100 ml. of 5% sodium hydroxide. Store in a refrigerator.

40% Urea.

*Procedure.* Frozen sections of grain were prepared as described under II, p. 93, in our earlier publication (5). Acetic alcohol-formalin fixative was used but found to offer no advantage over the absolute alcohol previously employed. The paraffin sections of root and acrospire were prepared as follows: Place for 1 hr. in each of the following solns., in the order given.

Acetic alcohol-formalin mixture.

95% ethyl alcohol.

45 ml. of 95% ethyl alcohol+55 ml. of *n*-butyl alcohol.

25 ml. of absolute ethyl alcohol+75 ml. of *n*-butyl alcohol.

*n*-butyl alcohol.

xylol.

The succeeding steps are identical with the corresponding ones given under B, pp. 92-93, in the previous paper (5). The staining reaction was carried out in the following steps:

1. Treat the sections on the glass slides for 10-15 minutes with a mixture of 5 vols. of the  $\alpha$ -naphthol soln., 5 vols. of 4% sodium hydroxide and 2 vols. of 40% urea, kept at 0-5°C. by immersing the container in an ice bath.

2. Add 20 vols. of 2% hypobromite and, after 3 min., stir in 2 vols. of 40% urea, and then 2 vols. of hypobromite. The maximum color develops in 3-5 min.

3. Transfer to glycerine, and after 2-3 min. to fresh glycerine.

4. Mount in glycerine. The reddish-orange color characterizing the positive reaction fades very gradually over a period of weeks.

## DISCUSSION

Photomicrographs of sections stained for arginine are shown in Figs. 1-4. The stain probably represents the distribution of protein

containing arginine since the amino acid is predominantly, if not entirely, a constituent of protein in this case. From Fig. 1 it is evident that no reaction for arginine appears in the pericarp of the wheat grain. The innermost of these layers is the testa layer, and the darkening in this region is not due to the arginine reaction but to the brownish-yellow pigmentation and high refractive index that this layer normally has. The cell walls of the large aleurone cells also are essentially negative. The degree of the positive reddish-orange reaction in both cytoplasm and nuclei of the aleurone cells is apparent.



FIG. 1

Arginine Reaction in the Outer Portions of a Section of Wheat Grain

The darkening in the testa layer is due to natural pigment and the high refractive index of this layer and does not represent a positive reaction. The only positive reaction is found in the aleurone cells which stand out prominently. 325 $\times$ .

The reaction in the endosperm was too faint to justify conclusions as to the distribution of arginine in definite structures, although one might expect to find this amino acid in the protein network of the endosperm. Cox, MacMasters and Hilbert (6) demonstrated the protein network in the endosperm of corn by the use of iodine staining of sections, combined with acid treatment to gelatinize the starch granules and thus facilitate their removal. Percival's (7) and Fairclough's (8) description of the protein distribution in the wheat endosperm is the same as that found by Cox *et al.* in corn, but nothing was said

concerning the manner by which the structure was demonstrated in the case of wheat.

The section of embryo shown in Fig. 2 indicates widespread distribution of arginine in these cells. The amino acid appears to be more concentrated in the nuclei than in the cytoplasm. The primary root structure, lower center, has taken little of the stain, while the branch roots, right and left center, which develop into the first pair of lateral



FIG. 2

Arginine Reaction in a Section of Wheat Embryo

The light area, lower center, is the primary root. Unstained control sections are practically invisible under the microscope. 50X.

roots on germination, and the base of the epicotyl, upper center, are strongly reactive. On the left of the epicotyl, Fig. 3, may be seen a strip of scutellum, the outer cells of which are darker. This is due to the stronger stain taken by these cells, and it should be mentioned that the color is not only more intense but it is redder and less orange than that in the scutellar region adjacent to the embryo. The stain in the

embryo itself has the same color and intensity as in the outer cells of the scutellum.

It may be seen that the epicotyl in Fig. 3 has a lighter-staining coleoptile while the inner leaves reacted more positively. The nuclei of the coleoptile cells, the cell walls and intercellular substance stained strongly but the cytoplasm appears to be poor in arginine; this may be

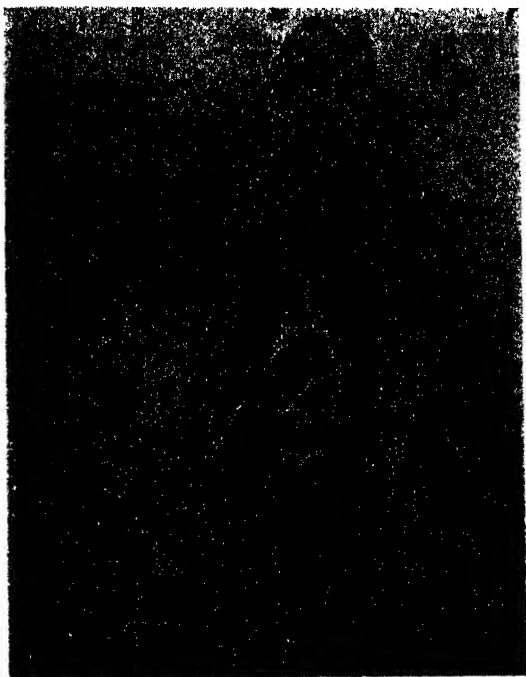


FIG. 3.

Arginine Reaction in a Section of the Epicotyl of Sprouted Wheat, and in the Scutellum, Which is the Strip of Tissue on the Left

Unstained control sections are practically invisible under the microscope. 49X.

due to the fact that the leaf cells contain more cytoplasm than the coleoptile cells and, hence, it may not be indicative of differences in cytoplasm *per se*.

The cross section of the root shown in Fig. 4 was taken in the center of the enlargement that occurs at the tip of the root. The peripheral

layer of cells is strongly reactive while the inner cells have a lighter, more diffusely stained, appearance.

Block and Bolling (9), pp. 77, 306, have recorded the results of arginine analyses on whole wheat, germ, bran, shorts, flour, gluten, gliadin, glutelin and glutenin. Arginine was present in all cases, occurring in a higher concentration in germ than in any other of the milled fractions. While the strong Serra reactions which were obtained in

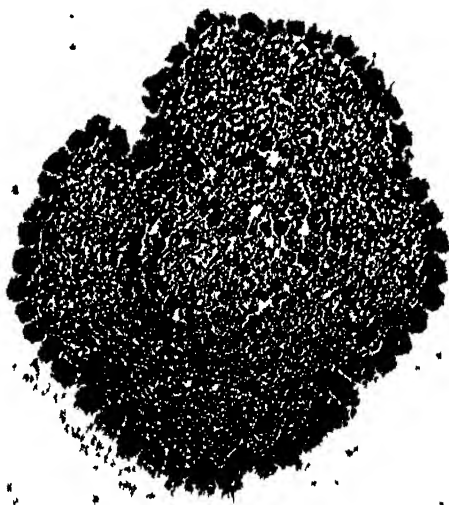


FIG. 4

Arginine Reaction in a Cross Section of Root Tip from Sprouted Wheat  
Control sections are practically invisible. 160 $\times$ .

certain histological loci are indicative of a relatively high concentration of arginine in these structures, the fact that the amino acid could not be detected in the pericarp of the grain, and was only weakly apparent in the endosperm, does not exclude the possibility of the presence of arginine in these regions. However, it is likely that, if arginine is present at these sites, it occurs in a rather low concentration.

#### ACKNOWLEDGMENT

The authors wish to thank Dr. A. O. Dahl of the Botany Dept., University of Minnesota, for his helpful criticisms of the manuscript.

## SUMMARY

Serra's histochemical arginine reaction was applied to microtome sections of wheat and the roots and epicotyl of the sprouted grain, and a description of the results obtained was given.

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# Investigations of Amino Acids, Peptides and Proteins. XXVIII. The Histidine Content of Casein\*

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## INTRODUCTION

Considerable evidence has been presented to indicate that amino acids in blood, urine and protein hydrolyzates may be determined conveniently by microbiological methods. Although, frequently, the amino acid data reported have not been highly accurate, they have been considered satisfactory for clinical and nutritional purposes. It is evident, on the other hand, that only amino acid values of the highest possible accuracy are useful for the determination of the amino acid composition, molecular weights and other characteristics of purified proteins. It seemed desirable, therefore, to ascertain the degree of accuracy attainable in the determination of amino acids by microbiological procedures and the results obtained from studies of the determination of histidine in amino acid test mixtures and casein are presented in this paper.

It was assumed that quantitative accuracy is possible only if a) the amino acid is not synthesized by the microorganism under the selected assay conditions, b) the microorganism is cultured under conditions such that biological variation<sup>1</sup> is prevented or minimized, c) sufficiently

\* For Paper XXVII in this series see Dunn *et al.* (1). The subject matter of this paper has been undertaken in cooperation with the Quartermaster Corps Committee on Food Research. This work was aided by grants from the American Home Products Company, Merck and Company, Inc., the Nutrition Foundation, Inc., Schering and Glatz and the University of California. The authors are indebted to H. Block, M. N. Camien and S. Shankman for technical assistance and valuable suggestions.

<sup>1</sup> The incitants to bacterial dissociation listed by Hadley (2) include temperature, food substances, oxygen tension, antiseptics, metabolic products and other factors. The possibility that a microorganism might exhibit variable response in the assay



precise experimental techniques are employed, d) the composition of the basal medium is such that the potential stimulatory or inhibitory influences of substances other than the test amino acid are almost completely nullified, and e) growth or acid production and concentration of the test amino acid present in the sample are directly proportional in the region of maximum slope of the standard curve.

### EXPERIMENTAL

*Leuconostoc mesenteroides* P-60 was carried on yeast-dextrose agar (Difco) by weekly transfers. The inoculum suspension was prepared by inoculating 20-ml. volumes of a culture medium<sup>2</sup> in 50-ml. Pyrex centrifuge tubes with *L. mesenteroides* slabs, incubating the suspension for 24 hours at 35°C., centrifuging the suspension and washing the cells twice by centrifuging them with 20-ml. portions of sterile saline. The washed cells were suspended in a volume of sterile saline equal to about four times that of the original cell suspension.

The basal medium (Medium D, Table I), containing 0.3 per cent NaCl, given in paper XVIII (6) was employed since it had been shown in experiments described in Papers XVIII and XXI (7) that histidine was not synthesized in 6 days and that acid production was nearly linear under a variety of conditions up to about 25  $\gamma$  of *l*(-)-histidine per tube. All of the components used to prepare the basal medium were

of an amino acid is indicated by the report of Kohn and Harris (3) that a strain of *E. coli* had been "trained" to require methionine and the report of Wright and Skeggs (4) that *L. arabinosus* had been "trained" to grow in the absence of tryptophan. It appears, however, that microbial variability may not be a significant factor in amino acid assays since no change has been observed in the writers' laboratory in the amino acid requirements of cultures which have been carried many months on media adequate to promote abundant growth.

<sup>2</sup> The culture medium was prepared by dissolving 75 ml. of an acid hydrolyzate of casein (prepared as described below), 0.200 g. of *l*(-)-cysteine hydrochloride, 0.050 g. of *l*(-)-tryptophan, 20.0 g. of glucose, 20.0 g. of sodium acetate  $\cdot 3\text{H}_2\text{O}$ , 5.0 ml. of Salts A solution (25 g. each of  $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  dissolved and made up with distilled water to 250 ml.), 5.0 ml. of Salts B solution (10 g. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.50 g. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.50 g. of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  dissolved and made up with distilled water to 250 ml.), 5.0 ml. of folic acid solution prepared according to the procedure of Hutchings *et al.* (5) and 10 ml. of AGU solution (0.660 g. of adenine  $\cdot 2\text{H}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$ , 0.600 g. of guanine  $\cdot \text{HCl}$  and 0.600 g. of uracil dissolved and made up with 0.5 *N* HCl to 500 ml.). This solution was diluted to 1 l. with distilled water and adjusted to pH 6.8 with the aid of a pH meter or bromothymol blue indicator.

The acid hydrolyzate of casein employed in the culture medium was prepared by refluxing a mixture of 200 g. of commercial vitamin-free casein and 2 l. of 6 *N* HCl for 24 hours. The residual solution, evaporated to remove excess HCl and decolorized with Nuchar XXX, was diluted to 2 l. and preserved in a glass-stoppered bottle in the refrigerator. It has been found that this solution was stable for at least three months.

of c.p. or higher quality and the sample of analytically pure *l*(-)-histidine monohydrochloride monohydrate, described in Paper IX (8), was employed as standard. The volumes of standard solutions of this histidine were measured with certified or calibrated flasks and pipettes corrected for temperature.

The basal medium, distilled water, sterile suspensions of the inoculum cultures, standard solutions of histidine and the acid hydrolyzate of casein were transferred to test tubes or flasks by means of a Brewer automatic pipet (Baltimore Biological Laboratories). The syringe assembly of the pipet was sterilized when required. The volume of each aliquot, determined by measuring the number of "shots" required to fill a calibrated volumetric flask to the mark, was checked 2 or 3 times during the course of each experiment. The tubes and flasks, placed in metal racks covered snugly with double-layer caps of clean toweling, were incubated in an air thermostat at 35°C. The response of the microorganism was measured by electrometric titration, pH and turbidimetric procedures. A mixture of amino acids, thoroughly ground in a ball mill and used in the basal medium, was found to be stable over a period of several months (67). In most of the experiments, no extra salt was added to the basal medium to compensate for that present in the neutralized aliquot of acid-hydrolyzed casein. Since it has been found previously that growth of *L. mesenteroides* P-60 was not appreciably affected by excess of salt smaller than about 100%, the approximately 10% excess in the present experiments was neglected.

## DISCUSSION

The data given in Table I support the view that the response of *L. mesenteroides* P-60 may be determined with high precision by electrometric titration, pH and turbidimetric methods. It may be noted, however, that the average values found in Rack 1 differed somewhat from those in Racks 2 and 3. Since the flasks in Rack 1 and those in Racks 2 and 3 were autoclaved separately, this possible source of variation was avoided in all subsequent experiments. It was considered probable that fluctuations in temperature might have been responsible for the observed variable response of the microorganism since the temperature of the air thermostat, at 8 positions adjacent to the walls near the top and bottom, was found to vary from 34.2 to 35.3°C. Measures taken in subsequent experiments to maintain the solutions at the same (not necessarily constant) temperature included preheating the solutions to 35°C. before inoculating them, mounting each rack on stilts to permit free circulation of air and shifting the racks to provide nearly equal exposure to direct currents of air from the fans and the heating elements.

The reproducibility of the histidine values found in five replicate tubes at each of 14 levels ranging from 1.59 to 22.37  $\gamma$  of *l*(-)-histidine in standard solutions of this amino acid is indicated by the data in

TABLE I

*Reproducibility of a Single Point in the Determination of Histidine  
with L. mesenteroides\**

Notation	Aliquots of single solution		Individual solutions							
	Electrometric		Electrometric titration**		pH			Optical density $\times 10$		
	titra- tion**	pH	Rack 1	Rack 3	Rack 1	Rack 2	Rack 3	Rack 1	Rack 2	Rack 3
(No. tubes)	(12)	(12)	(64)	(15)	(71)	(72)	(72)	(61)	(72)	(72)
Average	3.70	5.12	3.85	4.18	5.02	4.95	4.91	3.04	3.44	3.37
Median	3.70	5.12	3.85	4.15	5.02	4.95	4.91	3.05	3.41	3.38
Mode	3.70	5.12	3.94	4.15	5.00	4.95	4.93	3.00	3.40	3.38
MDM	0.027	0.003	0.13	0.07	0.03	0.04	0.04	0.06	0.10	0.13
p	0.029	0.004	0.11	0.04	0.02	0.03	0.03	0.05	0.09	0.11
P	0.008	0.001	0.01	0.01	0.002	0.004	0.004	0.006	0.01	0.01

\* All assays were conducted in 25-ml. conical flasks. Each aliquot or individual solution assayed contained the same quantity (approximately 5 $\gamma$ ) of l(-)-histidine/10 ml. of basal medium (Medium D, Table I) given in Paper XVIII (6). The solutions were incubated for 5 days at 35°C. in an air thermostat. The flasks in Rack 1 and those in Racks 2 and 3 were autoclaved at different times.

MDM—Average deviation from the mean.

p—Probable error of a single value.

P—Probable error of the mean.

\*\* Volume of 0.10 N NaOH.

Table II. Close agreement was found between the electrometric titration values for the five replicate tubes at each level of histidine in Rack C, as well as for the average values at a given level of histidine in Racks B and C. The titration values found for the replicate tubes in Rack A were, on the average, slightly higher at levels up to about 9  $\gamma$  of histidine and were somewhat lower at the higher levels up to about 20  $\gamma$  of histidine than the values at the corresponding levels for Racks B and C. Since the volume of inoculum added to each tube in Rack A was twice that added to each tube in Racks B and C, it was concluded that all solutions employed in an amino acid assay should be inoculated with the same volume of the same inoculum suspension.

The mean deviations from the mean of the titration values in Rack C, Table II, ranged from 0.02 to 0.07 and averaged 0.035. Such uni-

TABLE II  
*Reproducibility of Values Found in the Determination of Histidine  
 with L. mesenteroides\**

l(-)-Histidine per tube	Volume of 0.10 N NaOH to titrate solution								
	Rack C							Rack B	Rack A
	1	2	3	4	5	Ave.	MDM	Average <sup>a</sup>	Average <sup>b</sup>
$\gamma$	ml.	ml.	ml.	ml.	ml.	ml.			
0.00	0.86	0.80	0.84	0.84	0.88	0.84	0.02	0.73	0.82
1.59	1.85	1.89	1.86	1.86	1.90	1.87	0.02	1.84	1.90
3.18	2.78	2.69	2.77	2.78	2.75	2.77	0.02	2.76	2.86
4.77	3.69	3.76	3.81	3.81	3.78	3.77	0.03	3.69	3.83
6.36	4.68	4.64	4.70	4.66	4.65	4.67	0.02	4.63	4.66
7.97	5.49	5.54	5.63	5.47	5.49	5.52	0.05	5.49	5.60
9.56	6.34	6.29	6.29	6.22	6.35	6.30	0.04	6.29	6.37
11.15	7.06	7.00	7.00	7.02	7.02	7.02	0.02	7.10	7.02
12.74		7.75	7.65	7.80	7.72	7.73	0.04	7.82	7.71
14.33	8.35	8.40	8.36	8.35	8.37	8.37	0.02	8.39	8.18
15.91	9.06	9.20	9.05	9.16	9.15	9.12	0.06	9.08	8.94
17.50	9.79	9.60	9.69	9.64	9.56	9.66	0.07	9.67	9.48
19.09	10.16	10.24		10.15	10.17	10.18	0.03	10.26	10.15
20.68	10.83	10.79	10.74	10.72	10.77	10.77	0.05	10.86	10.57
22.27	11.22	11.33		11.24	11.29	11.27	0.04	11.34	11.05

\* The histidine solutions assayed contained the stated quantities of l(-)-histidine/10 ml. of Basal Medium D, Table I, given in Paper XVIII (6). All tubes (19×150 mm. Pyrex) were autoclaved at the same time and were incubated for 5 days at 35°C. in an air thermostat.

MDM Mean deviation from the mean.

<sup>a</sup> Five replicate tubes.

<sup>b</sup> Five replicate tubes. The volume of inoculum suspension per tube was twice that in Racks B and C.

formity of response is opposed to the view that reproducibility of microbiological assays is unattainable because of some "uncontrollable" biological variability. It may be expected, instead, that a particular microbial strain, cultured under accurately controlled environmental conditions, would exhibit constant average performance because of the predominant number of similar type cells present in the assay solution.

The accuracy with which histidine was determined in an amino acid test mixture simulating the composition of casein is indicated by the data given in Tables III-V. The average recoveries of histidine

TABLE III

*Assay of Histidine in Test Mixture Simulating the Composition of Casein by Electrometric Titration of Acid Produced by L. mesenteroides P-60*

I(—)-Histidine present per tube <sup>a</sup>	Flasks <sup>b</sup>			Test tubes <sup>c</sup>					
	Incubation time, hrs.			Incubation time, hrs.					
	60	84	109	74	74	96	96	120	120
$\gamma$	A	A	A	A	B	A	B	A	B
4.99	98.2	103.2	96.2	100.8	100.6	100.2	102.6	100.8	101.4
9.98	89.2	107.2	87.7	101.0	101.0	98.7	102.2	100.0	103.3
14.96	78.9	93.6	85.6	100.3	98.1	99.3	102.7	99.6	99.3
19.95	66.7	98.8	81.7	100.1	100.7	100.0	100.9	99.0	98.6
24.94	63.4	87.5	77.5	98.7	100.2	99.4	102.6	101.0	99.1
Average	79.3	98.1	85.7	100.2	100.1	99.5	102.2	100.1	100.3

<sup>a</sup> Quadruplicate standards and assays. Assays made with a Beckmann pH meter, Model M, checked against standard potassium acid phthalate.

<sup>b</sup> 25-ml. conical Pyrex flasks. Final volume of solution per flask was 10 ml.

<sup>c</sup> 19×150 mm. Pyrex test tubes. Final volume of solution per test tube was 10 ml. Recoveries of histidine at five levels ranging from 0.748 to 3.74  $\gamma$  of histidine per tube were 101 (96–105), 95 (93–99) and 100 (98–101)% at 46, 70 and 91 hr. incubation times, respectively. Recoveries of histidine at four levels ranging from 4.99 to 19.95  $\gamma$  per 13×100 mm. test tube (containing 3 ml. final volume of solution per tube) averaged 100 (97–103)%.

A—Medium not compensated for amino acids.

B—Medium compensated for amino acids. The composition of the medium was adjusted so that the ratio of the concentration of histidine to that of the total amino acids was constant at the different levels of histidine.

ranged from 99.5 to 100.3% under the most favorable experimental conditions (10-ml. volumes of media not compensated for amino acids in 19×150 mm. test tubes, 74 to 120 hours incubation time and electrometric titration of the acid produced by *L. mesenteroides* P-60). The recoveries of histidine were less satisfactory under the other conditions stated in the tables.<sup>3</sup>

Data showing the reproducibility with which histidine may be determined in casein are given in Table VII. The percentages of histidine

<sup>3</sup> Isbell (9) has shown recently that growth of *L. arabinosus* in relation to acid production was much greater when the medium contained suboptimal amounts of nicotinic acid.

found were closely agreeing at the five levels of casein even though the volume of inoculum suspension added to each standard and sample tube in Rack A was twice that added to each standard and sample tube in Rack B. As is shown in Table VIII, the histidine in caseins, obtained from two sources and hydrolyzed under four sets of conditions, averaged 2.94 (2.93–2.96)% calculated on an ash- and moisture-free basis while the histidine in caseins from two other sources averaged

TABLE IV

*Assay of Histidine in Test Mixture Simulating the Composition of Casein by pH Determination of the Acid Produced by L. mesenteroides P-60*

l(-)-Histidine present per tube <sup>a</sup>	Flasks <sup>b</sup>			Test tubes <sup>c</sup>					
	Incubation time, hrs.			Incubation time, hrs.					
	60	84	109	74		96		120	
$\gamma$	A	A	A	A	B	A	B	A	B
4.99	—	—	—	96.2	99.4	100.6	105.0	102.2	103.8
9.98	—	—	—	98.2	99.0	99.4	103.4	99.0	100.6
14.96	85.0	91.9	96.7	97.3	94.5	97.1	98.0	100.9	96.5
19.95	76.7	98.8	88.5	101.1	97.1	96.4	99.0	99.4	100.4
24.94	75.0	83.8	82.6	97.2	96.6	99.0	98.6	100.4	97.0
Average	78.9	91.5	89.3	98.0	97.3	98.5	100.8	100.4	99.7

<sup>a</sup> Quadruplicate standards and assays. Assays made with a Beckman pH meter, Model M, checked against standard potassium acid phthalate.

<sup>b</sup> 25-ml. conical Pyrex flasks. Final volume of solution per flask was 10 ml.

<sup>c</sup> 19×150 mm. Pyrex test tubes. Final volume of solution per tube was 10 ml. Recoveries of histidine at five levels ranging from 0.748 to 3.74  $\gamma$  of histidine per tube were 111 (101–116), 102 (93–108) and 108 (104–113)% at 46, 70 and 91 hr. incubation times, respectively. Recoveries of histidine at four levels ranging from 4.99 to 19.95  $\gamma$  per 13×100 mm. test tube (containing 3 ml. final volumes of solution per test tube) averaged 102 (96–106)% at 120 hours incubation time.

A—Medium not compensated for amino acids.

B—Medium compensated for amino acids.

2.85 (2.83–2.86)%. The histidine in three of these preparations averaged 3.03 (3.02–3.04)% while that in the fourth sample averaged 2.89 (2.90 and 2.88)% calculated to 16.0% nitrogen.

It would be difficult at best to determine the probable accuracy of these data if the commonly accepted view is correct that all samples

TABLE V

*Assay of Histidine in Test Mixture Simulating the Composition of Casein by Turbidimetric Determination of the Suspension Produced by L. mesenteroides P-60*

l(-)-Histidine present per tube <sup>a</sup>	Flasks <sup>b</sup>		Test tubes <sup>c</sup>					
	Incubation time, hrs.		Incubation time, hrs.					
	60	109	74	74	96	96	120	120
$\gamma$	A	A	A	B	A	B	A	B
4.99	100.2	104.2	101.8	100.2	100.2	102.6	104.8	104.8
9.98	97.5	98.7	101.6	98.0	104.0	100.6	99.4	100.8
14.96	93.0	97.7	102.7	97.4	101.5	101.5	102.4	100.4
19.95	91.3	96.8	102.1	100.4	102.1	99.1	102.0	99.1
24.94	97.4	96.4	102.3	98.2	104.4	97.0	105.5	102.8
Average	95.9	98.8	102.1	98.8	102.4	100.2	103.0	101.6

<sup>a</sup> Quadruplicate standards and assays. Assays made with a photoelectric colorimeter (Lumetron).

<sup>b</sup> 25-ml. conical Pyrex flasks. Final volume of solution per flask was 10 ml.

<sup>c</sup> 19×150 mm. Pyrex test tubes. Final volume of solution per tube was 10 ml. Recoveries of histidine at five levels ranging from 0.748 to 3.74  $\gamma$  of histidine per tube were 105 (72-119), 101 (89-108) and 102 (101-103)% at 46, 70 and 91 hrs. respectively.

A—Medium not compensated for amino acids.

B—Medium compensated for amino acids.

of casein, even the most highly purified preparations, are heterogenous.<sup>4</sup> Browne (22) has stated that the discovery that casein is a mixture of substances raises "serious questions about the adequacy of any of the methods so far described for preparing casein for scientific purposes" since there is no satisfactory basis "for deciding which of the separable components can properly be considered contaminants." On the other hand, the associates of Rogers (23) have expressed the belief that more

<sup>4</sup> Evidence for the non-homogeneity of casein has been presented by Danilewsky and Radenhausen (10), Osborne and Wakeman (11), Linderstrom-Lang (12-14), Carpenter *et al.* (15-18), Cherbuliez and Schneider (19), Mellander (20) and Warner (21) who have separated casein into fractions of different solubility, molecular weight, mobility, P-N and S-N ratios, amino acid composition (cystine, histidine, tryptophan and tyrosine) and antigenicity by extraction with alcoholic-HCl, by precipitation at different hydrogen ion concentrations and by ultracentrifugation.

evidence is needed before it can be concluded conclusively whether or not casein is a single protein or a mixture of substances.

This problem is complicated by the uncertainty concerning the true content of phosphorus, calcium and carbohydrate in casein. Hammarsten (24, 25) found in 1883 that casein, prepared by his method, contained 0.85 (0.83–0.88)% of phosphorus and later investigators (14, 18–21, 26–35) have reported approximately the same value (0.71–0.94). On the other hand, Ramsdell and Whittier (35) have shown recently that the casein-phosphorus complex, prepared by centrifuging skim milk and washing the deposit with water, contained 1.5% (0.67%

TABLE VI  
Percentage of Moisture, Ash and Nitrogen in Casein Samples

Casein sample	Moisture <sup>c</sup>	Ash <sup>c</sup>	Nitrogen <sup>b</sup>				
			Individual			Average	
						Uncorr.	Moist- and Ash-free
Authors <sup>a</sup>	6.21	0.55	14.50	14.44	14.54	14.49	15.55
SMA <sup>b</sup>	8.57	0.93	14.07	13.99	14.13	14.06	15.54
Commercial <sup>c</sup>	9.10	2.93	13.32	13.32	13.20	13.25	15.06
Vickery-Nolan <sup>d</sup>	5.89	1.88	14.53	14.57		14.55	15.78
Vickery-Nolan <sup>e</sup>	5.20	1.71	14.60	14.72	14.66	14.66	15.75

<sup>a</sup> Sample prepared by Dunn *et al.* (58) by isoelectric precipitation with HCl and washing with water, ethanol and diethyl ether.

<sup>b</sup> Vitamin-free sample obtained from the S.M.A. Corporation.

<sup>c</sup> Sample obtained from Braun Corporation, Los Angeles.

<sup>d</sup> Sample prepared by L. S. Nolan was obtained through the courtesy of Dr. H. B. Vickery. The identical material was employed previously by Vickery and White (59), Vickery (60) and Vickery and Winternitz (55). The analytical data given were obtained in the writers' laboratory.

<sup>e</sup> Sample same as that described in footnote d. The analytical data were furnished by Dr. H. B. Vickery. Vickery and White (59) reported 15.52% nitrogen on a moisture- and ash-free basis.

<sup>f</sup> Determined by drying to constant weight in a vacuum oven at 65°C.

<sup>g</sup> Determined by heating the air-dried material to constant weight in a muffle furnace at about 750°C.

<sup>h</sup> Determined by a semimicro Kjeldahl procedure. About 100 mg. samples were digested for 20 hrs. with 5 ml. of concentrated sulfuric acid containing 0.5 g. of powdered selenium, 250 g. of KHSO<sub>4</sub> and 5 g. of anhydrous CuSO<sub>4</sub> per liter.



organic and 0.86% inorganic) phosphorus. Casein containing as little as 0.29% phosphorus was prepared from skim milk which had been dialyzed for 36 days by Berggren (33) who stated that "there is nothing to indicate that this low value is the minimum phosphorus content of casein" and that "the results indicate that phosphorus is much more loosely bound in casein than has previously been supposed." The calcium content of casein preparations usually varies from 1.00 to 1.18% according to literature reports (21), although it may be reduced to less

TABLE VII

*Data Showing the Reproducibility of the Values Found for Histidine in Casein at Different Levels of Sample and in Duplicate Assays<sup>1</sup>*

Casein	Volume of base		Histidine found			
	Rack A	Rack B	Rack A	Rack B	Rack A	Rack B
$\gamma$	ml.	ml.	$\gamma$	$\gamma$	Per cent	Per cent
116.5	2.94	2.76	3.27	3.17	2.80	2.72
233.0	4.81	4.64	6.55	6.48	2.81	2.78
349.5	6.38	6.33	9.65	9.70	2.76	2.77
466.0	7.62	7.72	12.53	12.60	2.70	2.70
582.5	8.78	8.92	15.50	15.52	2.66	2.66
Average . . . . .					2.75 $\pm$ 0.05	2.73 $\pm$ 0.04

\* Histidine was determined by electrometric titration with 0.10 *N* NaOH. Each titration figure is the average of the values obtained for duplicate tubes in Rack A and triplicate tubes in Rack B. Histidine in the tubes of Rack A was determined by means of a standard curve constructed from the titration data obtained for the standard tubes incubated in Rack A. Histidine in the tubes of Rack B was determined similarly from the standard curve of Rack B. The volume of the inoculum suspension added to each tube in Rack A was twice that added to each tube in Rack B.

than 0.1% in casein preparations which have been treated with an ammoniacal solution of ammonium oxalate by the Van Slyke and Bosworth method (28, 36). Although it has been reported that Hammarsten casein (37, 38) contained 0.31–0.36%, Harris casein (39) 0.6% and Labco casein (39) 0.5% of galactose (or an equimolar mixture of glucose and mannose), Gortner (40) has stated that "It is questionable if this is an integral part of the casein molecule; more likely it is adsorbed by the casein during preparation."

The ash content of casein has been commonly determined by heating a sample to constant weight at 550°C. or higher temperatures, but it seems highly probable that some phosphorus is lost by this procedure, since it would be fortuitous if the ratio of calcium and phosphorus

TABLE VIII  
*Percentage of Histidine in Casein*

Casein sample		Hydrolysis conditions <sup>1</sup>	Histidine percentage <sup>1</sup>					
			Moisture- and ash-free basis			Calculated to 16.0% nitrogen		
			Assay 1	Assay 2	Average	Assay 1	Assay 2	Average
1	Authors	A	2.96±0.04	2.93±0.06	2.94	3.05±0.05	3.02±0.07	3.03
2	Authors	B	2.91±0.05	2.95±0.05	2.93	2.99±0.05	3.04±0.06	3.02
3	Authors	C	2.92±0.03	2.99±0.06	2.96	3.00±0.03	3.08±0.07	3.04
4	Authors	D	2.94±0.05		2.94	3.03±0.05		3.03
5	SMA	C	2.92±0.03	2.97±0.06	2.94	3.00±0.03	3.06±0.08	3.03
6	Commercial	A	2.82±0.03	2.90±0.09	2.86	2.99±0.04	3.08±0.10	3.04
7	Vickery-Nolan <sup>a</sup>	C	2.82±0.04	2.89±0.11	2.86	2.86±0.05	2.94±0.11	2.90
8	Vickery-Nolan <sup>b</sup>	C	2.79±0.04	2.87±0.11	2.83	2.84±0.05	2.91±0.11	2.88

\* Each value listed is the average found at five levels of sample. The standard curve employed in each assay was derived from the average titrations obtained with five replicate solutions of histidine.

\*\* Samples of casein varying from about 5 to 10 g. were accurately weighed and transferred quantitatively to standard-taper round-bottom flasks of 200 or 500 ml. capacity. Either 90 or 150 ml. of concentrated c.p. HCl were added, the mixture was allowed to stand 24 hours and the resulting solution was refluxed for 24 hours in an oil bath at 135–140°C. All of the hydrolyzates, except that containing starch, were opaque and colored light brown.

A—Hydrolysis carried out in air.

B—A weight of corn starch equal to that of casein was added and the mixture was hydrolyzed in air. The hydrolyzate was colored black.

C—Hydrolysis carried out under nitrogen. Oxygen-free nitrogen, prepared by passing nitrogen from a cylinder through a solution of chromous chloride, was passed for 48 hours through the flask, containing only the casein sample, and the condenser prior to the addition of the HCl. The purified nitrogen gas was run through the acid solution during the refluxing process.

D—Hydrolysis carried out under hydrogen made oxygen-free as described under C.

<sup>a</sup> Amino acid values calculated on the basis of the writers' moisture, ash and nitrogen data (Table VI).

<sup>b</sup> Amino acid values calculated on the basis of the moisture, ash and nitrogen data furnished by Dr. H. B. Vickery (Table VI).

present as calcium caseinate, phosphocasein,<sup>5</sup>  $\text{CaHPO}_4$ ,  $\text{Ca}_2\text{P}_2\text{O}_7$  (21),  $\text{Ca}_3\text{P}_2\text{O}_8$  (35) or  $\text{Ca}_3(\text{PO}_4)_2$  (42) were such that there would be no volatilization of  $\text{P}_2\text{O}_5$  under these conditions. Warner (21) has recommended recently, on the basis of the studies of Wichmann (43) and St. John and Midgley (44), that calcium acetate sufficient to bind all

TABLE IX  
*Amino Acid Sensitivity of Microorganisms*

Microorganism	Amino acid	Volume of base at half maximum*	Amino acid at half maximum	Sensitivity index** 100	Reference
		ml.	$\gamma$		
<i>L. arabinosus</i> 17-5	tryptophan	4.5	4.7	98	Greene and Black (61)
<i>L. arabinosus</i> 17-5	cystine	3.2	6.9	46	Dunn <i>et al.</i> <sup>*</sup>
<i>L. mesenteroides</i> P-60	histidine	6.6	16.5	40	Dunn <i>et al.</i> <sup>*</sup>
<i>L. fermenti</i> 36	methionine	2.1	9.0	22	Dunn <i>et al.</i> (62)
<i>L. mesenteroides</i> P-60	proline	6.2	28	22	Dunn <i>et al.</i> (6)
<i>L. mesenteroides</i> P-60	tyrosine	6.5	37	18	Dunn <i>et al.</i> (6)
<i>L. buchneri</i>	lysine	1.8	10	18	Wood <i>et al.</i> (63)
<i>S. faecalis</i>	threonine	5.0	38	17	Greenhut <i>et al.</i> (64)
<i>L. mesenteroides</i> P-60	leucine	6.8	42	16	Dunn <i>et al.</i> (6)
<i>L. arabinosus</i> 17-5	valine	6.5	45	14	Schweigert <i>et al.</i> (65)
<i>S. faecalis</i>	arginine	2.6	25	11	Stokes <i>et al.</i> (66)
<i>L. mannitolpoeus</i>	aspartic acid	1.4	13	11	Wood <i>et al.</i> (63)
<i>L. delbrückii</i>	phenylalanine	2.3	21	11	Stokes <i>et al.</i> (66)
<i>L. mannitolpoeus</i>	serine	2.0	19	10	Wood <i>et al.</i> (63)
<i>L. buchneri</i>	alanine	2	22	9	Wood <i>et al.</i> (63)
<i>S. faecalis</i>	isoleucine	2.1	30	7	Stokes <i>et al.</i> (66)
<i>L. arabinosus</i> 17-5	glutamic acid	3.6	64	6	Dunn <i>et al.</i> (58)
<i>L. mesenteroides</i> P-60	glycine	6.9	135	5	Dunn <i>et al.</i> (6)

\* Corrected for blank value.

\*\* Equivalent to slope (ml. base/ $\gamma$  of amino acid at half maximum).

<sup>\*</sup> Unpublished data.

of the phosphorus and to provide a small excess, be added prior to the ashing of casein. Even with this precaution, the true ash content was found to be 0.36–0.38% for purified casein, 0.39–0.60% for  $\alpha$ -casein and 0.90% for  $\beta$ -casein.

<sup>5</sup> See Gortner (40, 41) for a discussion of the orthophosphoric acid esters of hydroxyamino acids which have been isolated from casein.

TABLE X  
*Slopes and Intercepts Calculated from Titration Data Obtained in Assay of  
 Casein Samples\* Hydrolyzed under Two Conditions*

Sample		Volume of 0.10 N NaOH	Slope†	Intercept*
Type	Per tube			
	$X$	$Y$	$m'$	$b$
Histidine standard	$\gamma$	ml.		
	0.00	0.73		
	1.59	1.88		1.01
	3.18	2.76	0.55	1.02
	4.77	3.69	0.58	1.07
	6.36	4.63	0.59	1.15
	7.97	5.43	0.50	1.07
	9.56	6.29	0.54	1.07
	11.15	7.10	0.51	1.01
Casein (sample 1, Table VIII)	110.6	2.72		1.00
	221.2	4.44	0.0155	0.99
	331.8	6.16	0.0155	1.00
Casein (sample 4, Table VIII)	116.5	2.76		0.99
	333.0	4.62	0.0160	1.08
	349.5	6.30	0.0144	0.99

\* ml. base/ $\gamma$  of sample.

\*\* Calculated from the equation,  $Y = mX + b$  (where  $Y$  = ml. of base,  $m$  = average slope,  $X$  =  $\gamma$  of sample and  $b$  = a constant).

Even though different samples of purified caseins contain widely varying percentages of calcium and phosphorus, it should be possible to determine accurately the percentage of an amino acid in the protein

*per se* on the basis of the ash- and moisture<sup>6</sup>-free material. It seems apparent, however, that most of the data reported in the literature are not dependable. For this reason, it seems preferable to calculate the percentage of amino acids in terms of the total nitrogen.<sup>7</sup> This procedure, recommended in a recent paper by Chibnall<sup>8</sup> (48), should yield reliable data now that conditions have been established (36, 49, 50) for the quantitative determination of total nitrogen provided no nitrogen-containing substance has been lost or gained in the purifica-

TABLE XI

*Percentages of Histidine in Casein Calculated to 16.0% Nitrogen by Different Methods*

Sample	Single-curve method		Slope-ratio method	Common-zero 5-point method	Average
	Sample range		Sample range	Sample range	
	100-600 $\gamma$	100-350 $\gamma$	100-350 $\gamma$	100-350 $\gamma$	
Casein (sample 1, Table VIII)	3.05	3.09	3.15	3.04	3.08
Casein (sample 4, Table VIII)	3.03	3.05	3.08	3.08	3.06
Average	3.04	3.07	3.12	3.06	3.07

tion of the protein. High temperatures, strongly alkaline solutions and denaturing solvents have been avoided previously, yet these precautions give no assurance that there has been no loss of nitrogen through mild degradation of the milk casein. On the other hand, extra nitrogen may have been gained by milk casein which has been "purified" by dissolving it in ammonia despite the assumption of investigators who have employed the Van Slyke and Bosworth (28) method that all of

<sup>6</sup> According to Brand and Kassell's (45) isotope dilution studies with water containing O<sup>18</sup>, moisture may be determined accurately by drying proteins to constant weight at 110°C. in air or in a partial vacuum.

<sup>7</sup> Percentages of amino acids have been calculated by Block and Bolling (46) for hypothetical proteins containing 16.0% of total nitrogen. Although this procedure is subject to the objections raised by Vickery and Clarke (47), it is more advantageous than any other at present available for comparing percentages of amino acids in foods.

<sup>8</sup> The writers are indebted to Professor Chibnall for his courtesy in providing them with a copy of his paper prior to its publication.

the ammonia, free and combined as ammonium caseinate (51, 52), has been removed. It may be significant that casein preparations (28, 36, 53) which were dissolved in ammonia during purification contained from 15.8 to 16.1% of nitrogen while the caseins purified with ammonia-free reagents contained from 15.5 to 15.7% nitrogen according to Hammarsten (24), Tangl (27), Osborne and Guest (54), Bleyer and Seidell (31), Chibnall *et al.* (49), Vickery and Winternitz (55), Ramsdell and Whittier (35), Warner (21) and other investigators referred to by these authors. It is not unlikely, however, that the disagreements in these total nitrogen percentages may be accounted for by probable errors in the determination of moisture, ash and total nitrogen.

While the percentage of an amino acid in a protein hydrolyzate may be determined most reliably at the present time by the isotope dilution procedure, evidence has been presented in this paper that nearly the true percentage of histidine in casein may be determined by a microbiological method. The most probable errors were minimized, if not eliminated, by a) employing quantitative techniques in all critical measurements; b) using a microorganism, *L. mesenteroides*, which requires relatively low concentrations of histidine for its growth in a basal medium containing the other essential growth factors; c) selecting a basal medium composed only of pure chemicals (except folic acid solution) and of such a composition that the microorganism would grow and produce acid to its maximum potentiality at a relatively low concentration of histidine with a minimum, if not the complete absence, of stimulation and inhibition by substances other than histidine; d) culturing the microorganism under uniform, controlled conditions; and e) using an analytically pure sample of histidine as standard.

It has been shown that, under these conditions, histidine was assayed with high precision and accuracy in a mixture of amino acids simulating the composition of casein and that histidine was assayed with high precision in an acid hydrolyzate of casein. The probable accuracy of the percentages of histidine found for four casein preparations (Table VIII) can only be estimated. Although percentages varying from  $2.85 \pm 0.013$  to  $2.94 \pm 0.005$  were obtained when these values were calculated on a moisture- and ash-free basis, the higher figure seems more dependable since it is the average of three percentages ranging from  $2.93 \pm 0.02$  to  $2.96 \pm 0.04$  found in five duplicate (except one) assays of two caseins hydrolyzed under four sets of conditions. On the other hand, the latter data probably are somewhat low owing to

undetermined carbohydrate and phosphorus lost during ashing of the samples.

The average percentages of histidine found were  $2.89 \pm 0.010$  for one preparation and  $3.03 \pm 0.005$  ( $3.02 \pm 0.03$  to  $3.04 \pm 0.10$ ) for three preparations hydrolyzed under four sets of conditions when these values were calculated on the basis of 16.0% nitrogen. While the higher average figure may be more dependable than the lower, it is highly probable that moisture- and ash-free casein contains less than 16.0% nitrogen. Although the corrected percentages of total nitrogen found for the four casein preparations probably are reasonably accurate,<sup>9</sup> the observed range from 15.06 to 15.78% (Table VI) probably may be ascribed principally to variations in carbohydrate and to the phosphorus retained during the ashing process. It appears impossible at the present time, therefore, to determine what percentage of total nitrogen to employ as the basis for determining the true percentage of any amino acid in casein.

It may be reasonable to assume, however, that the true value<sup>10</sup> for

<sup>9</sup> Essentially the same corrected values, 15.75 and 15.78, were found in Vickery's and the writers' laboratory for the percentage of nitrogen in the Vickery-Nolan casein preparation (Table VI). In addition, the following corrected values have been found for total nitrogen in a sample of purified glycine prepared from Illini soybean in Vickery's laboratory: 16.93%, analysis by C. S. Leavenworth in H. B. Vickery's laboratory; 16.97%, analysis by Lila Miller in H. B. Lewis's laboratory; and 16.99%, analysis by J. D. Murray in M. S. Dunn's laboratory. These data were obtained through the courtesy of Dr. H. B. Vickery.

<sup>10</sup> The assumption that the histidine in milk casein was not lost or degraded during the separation, purification and hydrolytic processes is borne out, apparently, by the close agreement in histidine percentages (Table VIII) found for casein preparations hydrolyzed under four sets of conditions. It is further assumed that histidine may be determined with high accuracy in an acid hydrolyzate of casein by the described microbiological procedure. It has been found in general that the accuracy with which an amino acid may be determined in protein hydrolyzates by microbiological methods is directly proportional to the percentage of the amino acid in the protein and is inversely proportional to the amount of the amino acid required for the microorganism to produce unit quantity of acid. That all of the known amino acids except cystine, glycine, hydroxyproline and tryptophan occur in higher percentages than histidine in casein is an unfavorable circumstance, whereas the observation (Table IX) that the quantity of histidine required to promote maximum response of *L. mesenteroides* P-60 is nearly as small as that of any other amino acids favored for this purpose by other microorganisms which have been investigated, is a favorable one.

It has been noted that assay data of low precision and accuracy result usually, if not invariably, when the standard and sample response curves are irregular, lack a

histidine in casein lies between 2.94 and 3.03% and that it may be very nearly 3.00%. If this conclusion is valid, the suggestion is offered that 3.00% of histidine be adopted as the standard to which all other amino acid values in casein be referred. Histidine is nearly ideal for this purpose since it occurs in practically all proteins and it may be determined with relatively high precision by microbiological methods. An obvious advantage of this plan is that the labor of determining moisture, true ash and total nitrogen in casein preparations and the uncertain reliability of the analytical values would be eliminated.

It is of interest that casein preparations, irrespective of their source, have been found to contain nearly the same percentage of methionine (57), as well as of histidine. This observation may indicate that dif-

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nearly linear lower segment or have relatively small slope. Such data are inaccurate because the precision of the titration is too low and acid production is influenced not only by the test amino acid but also by other substances.

An objective criterion by which to evaluate the assay data has been suggested recently by Wood (56) who has stated that the "single-curve method" employed previously not only is theoretically unsound but also may give, in practice, results which are in error. Wood has pointed out quite correctly that if the response of the sample is due solely to its content of pure nutrient factor provided by the standard, the curve relating dosage to response must be identical with the standard curve except that the scale is different. He has suggested that the ratio of scales (slopes) of straight-line curves with a common intercept is the only sound measure of the relative potency of two preparations. In practice, the intercepts of such curves may be determined by calculation from their straight-line equations or by extrapolation to zero dosage of the straight-line segments of the curves showing the relation of the response (ml. of base) to the dosage ( $\gamma$  of amino acid or sample).

It has been shown in Table X that the slopes and intercepts were nearly constant for nearly all of the points of the linear segment of the standard curve as well as of the author's casein sample hydrolyzed under two sets of conditions. Percentages of histidine in casein have been calculated by (a) the single-curve method, (b) the ratio of slopes of the standard and sample linear curves having a common intercept and (c) the common-zero 5-point design equations referred to in Wood's second paper. The calculated percentages (Table XI) of histidine are in good agreement and it follows that this is necessarily true in this case (and all other assays) where stimulatory and inhibitory substances are absent or are made ineffectual by the nullifying action of the basal medium. If intercepts which are in disagreement result from the assay data, recourse may be had to Wood's common-zero 5-point design equations in calculating an amino acid value which is correct within a certain limiting deviation. On the other hand, it appears that assay values of relatively high accuracy are attainable only with linear segments of the standard and sample curves which yield a common intercept. It is believed, furthermore, that this goal may best be reached through the use of near-optimal basal media and experimental conditions.



ferent samples of casein contain nearly constant proportions of  $\alpha$ - and  $\beta$ -casein or that these fractions contain nearly constant proportions of amino acids. That casein and its two fractions contain nearly the same corrected percentage of nitrogen (21) is not opposed to the latter hypothesis. Even though the usual casein preparations are non-homogenous, it may be that casein protein *per se* is definitely characterized by the types, proportions and distribution of its constituent amino acids.

### SUMMARY

Evidence has been presented which supports the view that histidine in amino acid test mixtures and in casein can be determined with *L. mesenteroides* P-60 with high precision and accuracy. Since nearly the same value was found for four casein samples hydrolyzed in the presence and absence of starch or oxygen, it has been concluded that histidine is inappreciably destroyed during acid hydrolysis. It has been suggested that the protein *per se* in different samples of casein may contain nearly the same percentage of histidine. It has been proposed that 3.00% of histidine in casein be adopted, tentatively, as the standard to which all other amino acid values be referred because of the difficulties inherent in the accurate determination of amino acids on other bases.

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# **The Excretion of B-Complex Vitamins by Normal Adults on a Restricted Intake**

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## **INTRODUCTION**

There is much interest in the role intestinal bacteria may play in the nutrition of man. Previously we have reported that bacteria in the intestine of normal adults on a normal diet presumably synthesize relatively large quantities of B-complex vitamins which are excreted in the feces (1). In this regard, other investigators report isolated studies indicating that thiamine, riboflavin, nicotinic acid and biotin synthesized by the intestinal flora can be utilized by human subjects on a restricted dietary intake (2, 3, 4, 5).

No reports have been found in the literature regarding coexisting dietary intake and the excretion in both urine and feces of several of the lesser-known B-complex vitamins in normal adults subsisting on a diet restricted in these vitamins. Data are reported in this paper for 5 normal young men fed, for 15 weeks, a diet containing 19-60 percent as much thiamine, riboflavin, niacin, biotin, *L. casei* factor, pantothenic

acid and pyridoxine as is contained in a "normal diet." In addition, the level of protein was low (40 g., only 3 g. of which were animal protein), and corn supplied 27% of the dietary calories. Two control subjects received the same diet supplemented with animal protein and synthetic B-vitamins.

### EXPERIMENTAL

A discussion of the subjects and details of urinary and fecal collections, as well as methods for the microbiological and chemical determinations of the B-vitamins, has been described in a previous paper (1) relating to the excretion of B-vitamins on a normal intake. Pyridoxine in the diet was determined microbiologically using *Saccharomyces carlsbergensis* according to the method of Atkin and co-workers (6).

All meals were prepared and served under the direct supervision of a trained dietitian. The menu was followed strictly and all portions were carefully weighed. Extra identical meals were prepared one day each week, carefully ground, homogenized, frozen and used for assay. The canned foods were purchased in one lot of the same pack in a quantity sufficient for the entire experiment. During an initial control period of 12 weeks a normal diet supplying liberal quantities of all nutrients was fed to all 7 subjects. The content of this diet in the B-vitamins under consideration is shown in Table I. The restricted diet provided daily about 3300 calories, 40 g. of protein, 380 g.

TABLE I  
*Intake of B-Vitamins*

Vitamin	Control Period (all 7 subjects)	First 5 weeks restricted period (all 7 subjects)	6th-15th week restricted period	
			5 rest'd subjects	2 suppl'd subjects
Thiamine, mg.	1.44	.53	.54	1.73
Riboflavin, mg.	1.84	.36	.32	1.98
Niacin, mg.	15.6	6.8	5.7	17.6
Biotin, $\gamma$	44	24	20	80
<i>L. casei</i> factor, $\gamma$	64	26	22	112
Pantothenic acid, mg.	4.7	1.3	1.1	7.2
Pyridoxine, mg.	1.76	1.12	1.05	4.05

of carbohydrate, 175 g. of fat, 200 mg. of calcium, 560 mg. of phosphorus, 12 mg. of iron, 90 mg. of ascorbic acid and 16,000 I.U. of vitamin A. The determined (by analysis) average daily food and supplemental content of the B-vitamins under consideration in this report is listed in Table I. These values represent maximum intake since consumption was not always 100%. The food consisted of foods frequently consumed in certain parts of the country, such as corn meal in various forms (as fried mush, muffins and cookies), spaghetti, carrots, string beans, beets, gelatin, apples, pears, pineapple, salt pork, oleomargarine and sugar.

For 5 weeks all 7 subjects ate the restricted diet with no supplementation. At the beginning of the sixth week, and thereafter through the fifteenth week, all subjects were given daily, by capsule, 700 mg. calcium, 540 mg. phosphorus, 20 mg. iron and 666 I.U. vitamin D. In addition, the two control subjects were given daily, in tablets, the following crystalline supplements divided equally among the three meals: 1.2 mg. thiamine hydrochloride, 1.5 mg. riboflavin, 12 mg. nicotinamide, 60  $\gamma$  biotin, 90  $\gamma$  synthetic *L. casei* factor, 6.6 mg. *dl* calcium pantothenate, 3.3 mg. *d* calcium pantothenate, 0.3 mg. *p*-aminobenzoic acid, 3 mg. pyridoxine hydrochloride and 1 g. choline dihydrogen citrate. Corresponding placebos were given to the five restricted subjects. In addition, the controls were given in their food 45 g./day of calcium caseinate (Casc).

### RESULTS

Daily intake and excretion (in the urine and feces) of the B-vitamins are shown in Figs. 1-8. Each value represents the group 24-hour average determined on a sample collected for four consecutive days each week. On one day of each week the food was analyzed to give the values listed in Table I as dietary intake. During the period of restricted intake the fecal excretion of all vitamins remained as high as, or was higher than, during the period on the normal diet. There was no

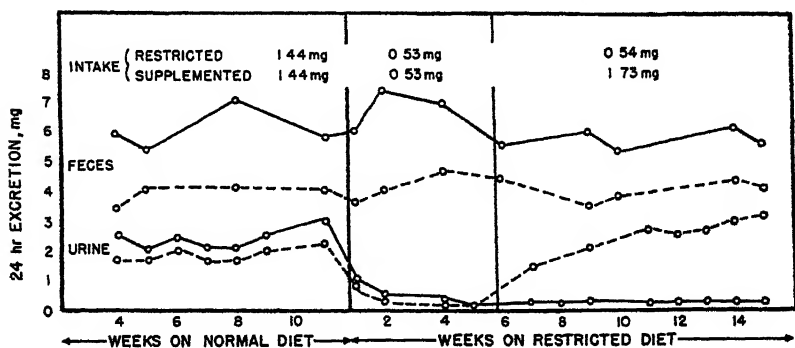


FIG. 1  
Thiamine Excretion

FIGS. 1-8, inclusive, present group average urine and feces excretions of various B-vitamins. The averages for the 2 supplemented subjects are shown by broken lines, and for the 5 restricted subjects by solid lines. The first of the two vertical lines represents the end of the normal diet and the beginning of the restricted diet. The second vertical line represents the time at which supplementation of the two men was begun.

It should be noted that in Figs. 7 and 8 the abscissae for feces values are higher than for urine. This was done in order to separate the curves which would otherwise have closely coincided.

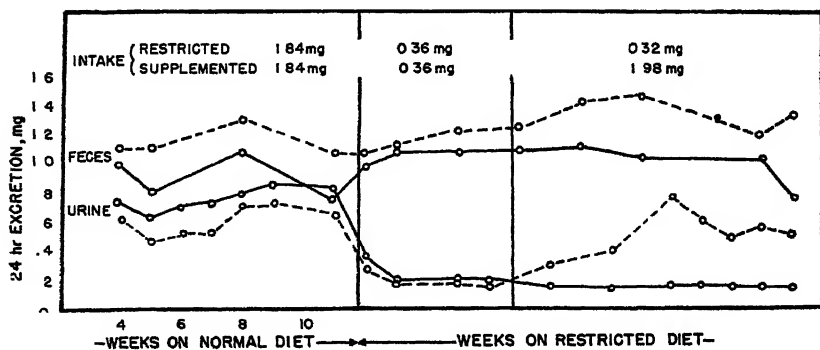


FIG. 2  
Riboflavin Excretion

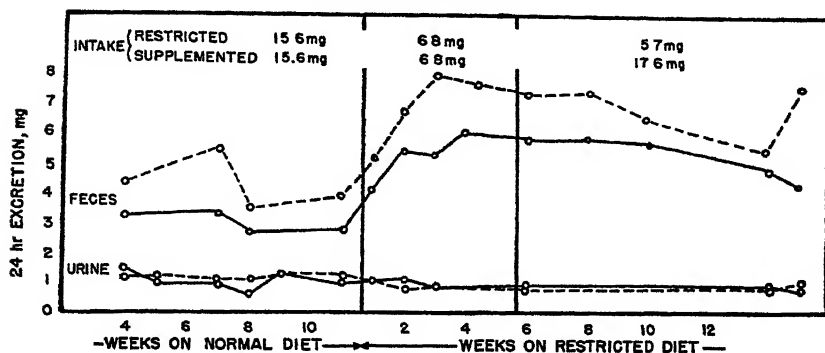


FIG. 3  
Niacin Excretion

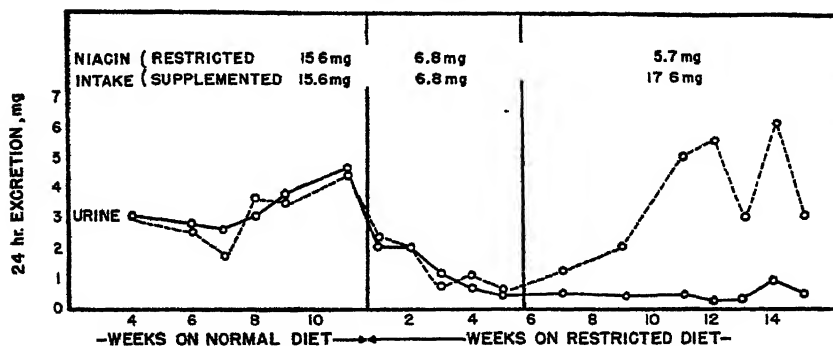


FIG. 4  
N-Methylnicotinamide Excretion

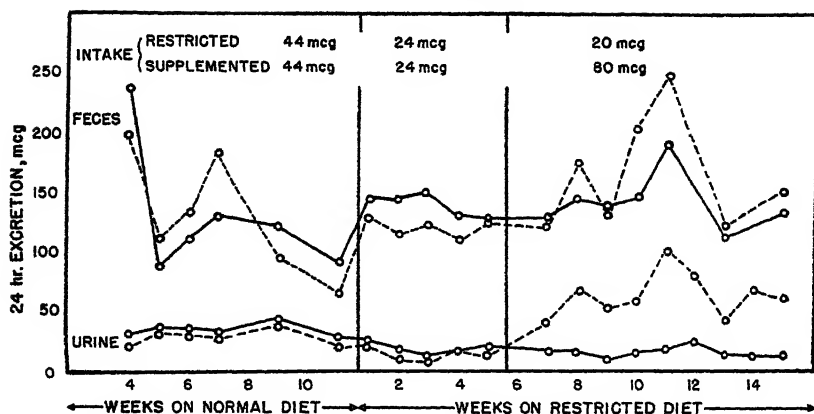


FIG. 5  
Biotin Excretion

evidence, except possibly in the case of *L. casei* factor, that supplementation with crystalline vitamins increased the fecal excretion (Note Figs. 1-8).

On the other hand, the urinary excretion of thiamine, riboflavin, N<sup>1</sup>-methylnicotinamide and pantothenic acid dropped quickly and markedly, the excretion of biotin and pyridoxine decreased to somewhat less than half their previous levels, and the excretion of niacin and *L. casei* factor decreased only slightly. When additional crystalline B-vitamins were provided to the supplemented subjects their urinary

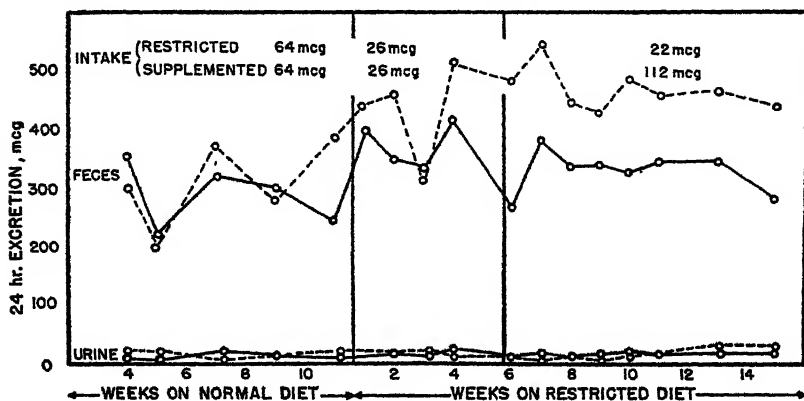


FIG. 6  
*L. Casei* Factor Excretion



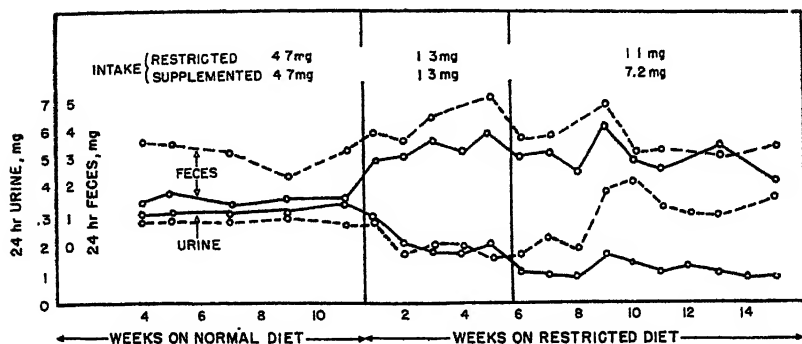


FIG. 7  
Pantothenic Acid Excretion

excretion of thiamine, riboflavin,  $N^1$ -methylnicotinamide, biotin, pantothenic acid and pyridoxine gradually returned to the levels of the control period. This was not true, however, of urinary niacin and *L. casei* factor, as vitamin supplementation did not affect their excretion. As was noted above, niacin and *L. casei* factor were also the two vitamins excretion of which decreased little, if any, from levels on a normal diet.

In Table II the average excretions of the various vitamins in the urine and feces of the restricted group are compared with their intakes. The data for the 11th.-15th. weeks, inclusive, on the restricted diet are

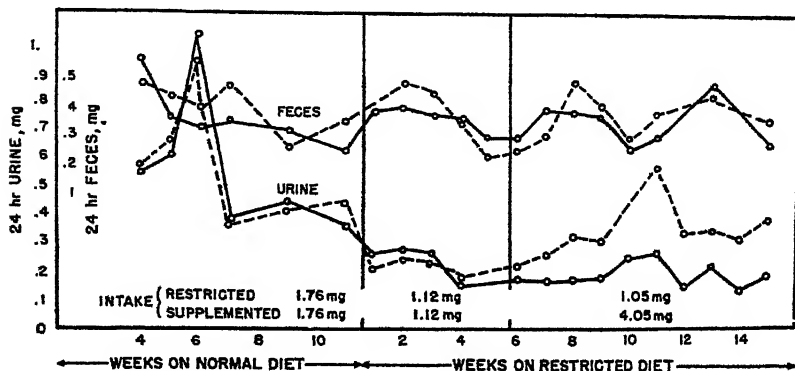


FIG. 8  
Pyridoxine Excretion

TABLE II

*(Comparison of Urine and Feces Excretions with Intake for the  
11th Week to 15th Week on a Restricted Diet)*

Vitamin	Means 5 men, 5 weeks			
	Intake	Urine	Feces	Urine plus feces
Thiamine, mg.	.54	.01	.56	.57
Riboflavin, mg.	.32	.16	.87	1.03
Niacin, mg.	5.7	.7	4.4	5.1
N <sup>1</sup> -methylnicotinamide, mg.	—	.4	—	—
Biotin, $\gamma$	20	17	148	165
<i>L. casei</i> factor, $\gamma$	22	3	322	325
Pantothenic acid, mg.	1.1	1.0	2.8	3.8
Pyridoxine, mg.	1.05	.20	.32	.52

used for this comparison. The group average urinary excretion of thiamine, niacin, N<sup>1</sup>-methylnicotinamide, *L. casei* factor and pyridoxine ranged from 2 to 19% of intake, while the urinary excretion of riboflavin was 50% of intake, and of biotin and pantothenic acid about equal to intake.

Fecal excretions of all vitamins except niacin and pyridoxine exceeded intake, biotin by 8-fold and *L. casei* factor by 15-fold. The combined urine and fecal excretion of all vitamins except pyridoxine approximated or greatly exceeded intake.

### DISCUSSION

Evidence is presented implying that intestinal bacteria synthesize large quantities of certain B-complex vitamins when healthy young adults subsist for 15 weeks on a restricted intake of these vitamins and of animal protein. Since the feces continued to contain relatively large amounts of these vitamins while the urinary excretion of most of them dropped markedly, it appears that only slight absorption could have occurred through the large intestine. It is possible that, in the case of certain vitamins, these quantities could be nutritionally significant on this restricted diet considering that the normal requirements may be very low. The fact that urinary excretion of pantothenic acid and biotin in the unsupplemented subjects approximated and on certain weeks exceeded dietary intake implies that the pantothenic acid and

biotin may have been absorbed through the large intestine or may have come from unidentified metabolic processes in the tissues.

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#### SUMMARY

1. Average daily urinary and fecal excretions of thiamine, riboflavin, nicotinic acid, biotin, *L. casei* factor, pantothenic acid and pyridoxine and urinary excretion of N<sup>1</sup>-methylnicotinamide are reported for five young normal adults on a restricted intake for 15 weeks. Values are also given for two subjects receiving supplements equivalent to, or in excess of, those levels found in the original normal diet.

2. Average fecal excretion of all vitamins was as high, or higher, on the restricted diet as on the normal diet.

3. Fecal excretion was unaffected by vitamin supplementation.

4. The average urinary excretion of thiamine, riboflavin, N<sup>1</sup>-methylnicotinamide and pantothenic acid dropped markedly on the restricted diet; biotin and pyridoxine dropped moderately; and niacin and *L. casei* factor dropped only slightly, if at all.

5. Urinary excretion of thiamine, riboflavin, N<sup>1</sup>-methylnicotinamide, biotin, pantothenic acid and pyridoxine returned to the original or to even higher levels following supplementation with crystalline vitamins. Urinary niacin and *L. casei* factor were not affected by supplementation.

6. Urinary excretion of biotin and pantothenic acid approximated intake on the restricted diet.

7. Fecal excretion of all vitamins except niacin and pyridoxine exceeded intake on the restricted diet.

8. The combined urinary and fecal excretion of all vitamins, except pyridoxine, approximated or greatly exceeded intake on the restricted diet.

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# Note on the Ability of Rat Liver Slices to Perform Certain Reactions After Different Times of Incubation

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## INTRODUCTION

When liver slices are shaken in Krebs' bicarbonate solution in an atmosphere of 95% oxygen and 5% carbon dioxide at 37°C. there is a progressive increase in the protein content of the fluid (1). Thirty per cent of this increase can be accounted for by erythrocytes which slowly work out of the capillaries, and by a few small clumps of liver cells which break off from the slice. The remaining 70% is due to protein which has diffused out of the cell. After 4 hours of incubation approximately 20% of all the protein originally present in the slice has diffused into the surrounding medium. This fact raises the question of whether cells which have lost protein in this way are still capable of carrying out certain synthetic reactions which do not occur when the cell structure has been completely destroyed. To answer this, the following experiments were made.

## EXPERIMENTAL

Rats were killed by a blow on the head and bled. Liver slices were made in the usual way and 300 mg. (wet weight) were suspended in 4.0 cc. Krebs' bicarbonate solution and equilibrated in 50 cc. Erlenmeyer flasks with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Eight flasks (four pairs) were subjected to continuous shaking in a water bath at 37°C. At zero time the substrate to be tested was added to one vessel by means of a syringe and needle through the rubber tubing. At the end of one hour the experimental vessel and its control were removed and 1.0 cc. of trichloroacetic acid added to each. The substrate was then added to another vessel and the slices were allowed to react with it during the second hour, after which it and a control were removed and trichloroacetic acid was added. This process was repeated for the third time and again for the fourth time. In this way, the rate of the reaction in the first, second, third and fourth hours of incubation was determined. Four reactions were studied.

The conjugation of 2.0 mg. of sulfanilamide which requires acetate (2); the conjugation of 1.0 mg. of morphine HCl which requires glucuronic acid (3); the production of a hydroxyphenyl compound from 4.0 mg. *DL*-phenylalanine (4); and the production of urea from 4.0 mg. of ammonium sulfate which involves a cyclic process. As urea is produced in appreciable quantities by the surviving liver slice, its accumulation would tend to inhibit the rate of urea formation from added ammonia, so a suitable amount of urea was added to all vessels before addition of the ammonium sulfate. Thus, the first pair of vessels received 0.3 mg. urea, the second pair 0.2 mg. and the third pair 0.1 mg. The added urea plus that formed by the slices was practically constant and thus a true estimate of the enzyme activity could be reached. The urea was estimated by the method of Ormsby (5). The other estimations were carried out by the methods described in the original papers.

Table I summarizes the results. All the reactions proceed at the same rate during the first and fourth hour of incubation despite the fact that during the latter period much more protein has been lost

TABLE I

*The Amounts of Urea Formed, Phenylalanine Hydroxylated, Sulfanilamide and Morphine Conjugated by 300 mg. of Rat Liver Slices during the First, Second, Third and Fourth Hours of Incubation at 37°C.  
in 95% O<sub>2</sub> and 5% CO<sub>2</sub>*

Time	Urea formed	Phenylalanine hydroxylated	Sulfanilamide conjugated	Morphine conjugated
	mg.	mg.	mg.	mg.
1st. hour	0.23	0.33	0.24	0.34
2nd. hour	0.25	0.33	0.29	0.36
3rd. hour	0.26	0.32	0.15	0.36
4th. hour	0.25	0.38	0.19	0.34

from the cell. There are three possible interpretations of this fact. The least probable is that the liver cell contains a considerable amount of protein which has no catalytic function. The second is that the cell has a large reserve and can, therefore, function apparently normally despite a 20% loss of the enzymes involved. In this respect it appears that most cells do contain succinoxidase in excess of their normal needs because addition of succinic acid to tissue slices always greatly increases the oxygen uptake. The third is that synthetic reactions can occur in partially disorganized cells if either, (a) essential energy producing reactions are still functioning or, (b) compounds of high energy value are added. In this respect Lippmann (6) has shown that sulfanilamide is acetylated by liver suspensions if adenosine triphosphate

is added. Whatever the explanation, the tissue slice technic allows the liver cells to function apparently normally, for certain reactions at least, during a period of four hours.

### SUMMARY

The rate of urea formation from ammonium salts, the rates of conjugation of sulfanilamide and morphine, and the rate of hydroxylation of phenylalanine are the same during the first and fourth hours of incubation of rat liver slices under the experimental conditions described.

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# Production of Citric Acid from Cane Molasses\*

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## INTRODUCTION

Commercially, citric acid is chiefly produced by the fermentation of beet molasses by fungi (5). Few references to the production of citric acid from cane molasses are found in the literature and the general impression prevails that low yields are obtained from this substrate. Cahn (1) has described a process in which bagasse and other supporting materials are impregnated with cane molasses or other fermentable carbohydrates. A similar process is discussed by Roberts and Murphy (11) in which the molasses was distributed on sphagnum moss and fermented. These processes seem to have had little commercial application. Another reference to the fermentation of cane molasses is given by Das Gupta *et al.* (2), where a 5% molasses solution was fermented by a mucor species to yield approximately 33% citric acid based on the sugar consumed.

Preliminary experiments in this laboratory (7) indicated that low yields of citric acid were obtained when cane molasses solutions were fermented by strains of *Aspergillus niger* normally producing high yields from synthetic and beet molasses media. An attempt has been made to investigate the character of the materials in the cane molasses responsible for these low yields, and to devise a process for removing these materials or eliminating their effect on the fermentation.

## EXPERIMENTAL

*Cultures, Media and Methods.* The cultural techniques and analytical methods were the same as those given in previous papers (8, 9). Two media based on previous experiments (4, 9) were used. A synthetic medium contained 140 g. purified sucrose (from the American Sugar Refining Co.); 2.25 g.  $\text{NH}_4\text{NO}_3$ ; 1.00 g.  $\text{KH}_2\text{PO}_4$ ; 0.25 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.1 mg. Fe (as  $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ); sufficient HCl to adjust the medium to pH 2.3; distilled water to 1 l. The beet molasses medium was prepared by diluting 280 g. of beet molasses (from the Michigan Beet Sugar Co.) to 1 l. with dis-

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\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

tilled water, adding 1.2 g.  $K_4Fe(CN)_6 \cdot 4H_2O$ , 2 ml. of 1 N  $H_2SO_4$ , and 10 g. of filter cell, and allowing precipitation to occur for 2 days at 10°C. The pH of the supernatant solution was approximately 6.5.<sup>1</sup> Of these media, 30 ml. portions were placed in flat 6 oz. bottles, inoculated and incubated in a horizontal position at 30°C. for the periods given in the tables.

Two cultures of *A. niger* were used in these experiments. *A. niger* 62 is the same as *A. niger* 67 used by Wells *et al.* (14), while *A. niger* 72 was received as a transfer of *A. niger* ATCC 1015. After several of the experiments had been concluded, it was found that the earlier high yields of citric acid obtained with *A. niger* 72 on synthetic media (55%) were no longer possible. Observation of the organism did not reveal any markedly different characteristics, except that the mycelium was invariably covered with spores during the fermentation, an occurrence which, in the past, had often been associated with low yields of citric acid. It was thought that the culture might have degenerated and a number of procedures were tried to obtain a normal citric acid-producing culture. In one of these procedures, the culture was plated on agar and well-isolated colonies were picked for further study. One of these strains designated as *A. niger* 72-4 was found to have approximately the same citric acid-producing ability as the normal parent culture. Approximately 90% of the acid produced by *A. niger* 62, and 75% of the acid produced by *A. niger* 72 and *A. niger* 72-4, was citric acid. The yields presented in the tables are the percentages of citric acid monohydrate ( $H_3C_6H_5O_7 \cdot H_2O$ ) calculated on the sucrose available for fermentation. Each figure is the average of at least 2 and often 5 replicates.

Preliminary experiments, as indicated in Table I, showed that substitution of Cuban High Test molasses for purified sucrose in the synthetic medium resulted in low yields of acid. Variation of the inorganic components of the medium did not change this result appreciably. Fermentation of mixtures of the synthetic medium and the molasses medium showed that very small amounts of the Cuban High Test medium could markedly reduce the yields of citric acid.

A similar experiment in which mixtures of beet molasses medium and Cuban High Test molasses medium were fermented by three

<sup>1</sup>The authors are indebted to Mr. William Eisenman of the Heyden Chemical Corporation for suggesting the potassium ferrocyanide treatment of molasses and for a statement of the procedure which he employed. A systematic study of various factors affecting yield of citric acid with the treatment is given by Gerhardt (4).

TABLE I  
*Acid Production from Mixtures of Synthetic Medium and  
 Cuban High Test Molasses Medium*

Synthetic medium	Cuban High Test Molasses medium*	Titratable acid, as citric
volume-per cent	volume-per cent	per cent
0	100	27
20	80	15
40	60	19
60	40	26
80	20	41
100	0	68

\* Cuban high test molasses medium: 180 g. molasses (140 g. sucrose); 2.25 g.  $\text{NH}_4\text{NO}_3$ ; 1.00 g.  $\text{KH}_2\text{PO}_4$ ; 0.25 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; distilled water to 1 l.

Organism: *Aspergillus niger* 62.

strains of *A. niger* is reported in Table II. Here again, the replacement of as little as 20% of the volume of beet molasses medium with cane molasses medium resulted in a marked decrease in acid production. Strain 72-4 seemed to be more sensitive than the other cultures, since

TABLE II  
*Acid Production from Mixtures of Beet Molasses Medium and  
 Cuban High Test Molasses Medium*

<i>Aspergillus niger</i> 62			<i>Aspergillus niger</i> 72			<i>Aspergillus niger</i> 72-4		
Beet molasses medium	Cuban high test molasses medium*	Titratable acid as citric	Beet molasses medium	Cuban high test molasses medium†	Titratable acid as citric	Beet molasses medium	Cuban high test molasses medium	Titratable acid as citric
volume-per cent	volume-per cent	per cent	volume-per cent	volume-per cent	per cent	volume-per cent	volume-per cent	per cent
100	0	125	100	0	130	100	0	82
80	20	106	80	20	75	98	2	56
60	40	39	60	40	38	96	4	47
40	60	35	40	60	30	94	6	39
20	80	31	20	80	29	92	8	35
0	100	27	0	100	34	91	9	32

\* Cuban high test molasses medium: 180 g. molasses; 2.25 g.  $\text{NH}_4\text{NO}_3$ ; 0.25 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; distilled water to 1 l.

† Cuban high test molasses medium: 180 g. molasses diluted to 1 l. with distilled water.

as little as 2 volume-% caused a marked decrease in the yield of citric acid.

It seemed possible that a part of this inhibitory effect might be due to the metallic ions present in the molasses, since metallic ions were found to exert a marked effect on the fermentation of synthetic media (9), and the Cuban High Test molasses sample contained 1.68% ash. Passage of the molasses over an ionic exchange material was tried as a means of removing these metallic ions. The results of one of these experiments are shown in Table III. The cation exchange treatment re-

TABLE III

*Treatment of Cuban High Test Molasses with Cation Exchange Materials*

Exchange material	Ash	Analysis of ash		Titratable acid, as citric
		SiO <sub>2</sub>	Ca	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
None	1.68	7.1	8.0	21
IR-100 <sup>+</sup>	0.24	38.5	1.8	35
Zio-Karb H <sup>†</sup>	0.35	32.0	1.3	23
Alkalax <sup>‡</sup>	0.88	46.0	3.8	42

\* Resinous Products and Chemical Co., Philadelphia.

† Permutit Co., New York.

‡ Research Products Co., Madison.

Medium: 180 g. treated molasses (140 g. sucrose); 1.00 g. KH<sub>2</sub>PO<sub>4</sub>; 0.25 g. MgSO<sub>4</sub> · 7H<sub>2</sub>O; 2.25 g. NH<sub>4</sub>NO<sub>3</sub>; distilled water to 1 l. adjusted to pH 6 with NH<sub>4</sub>OH.

Organism: *Aspergillus niger* 62.

duced the ash content of the molasses rather markedly, but did not result in increased acid production in all cases. It is possible that some of the inhibitory metallic ions were adsorbed on the silica, which of course is not strongly enough ionized in solution to be removed by this process, and hence remained in the treated molasses.

Several other partially successful treatments are tabulated in Table IV. Some improvement resulted from the ferrocyanide treatment of this molasses. This treatment would precipitate some of the iron and manganese which are present in the ash of the molasses.

A quantity of the Cuban High Test molasses was ashed at 510°C. Increasing amounts of this ash were added to the synthetic and beet molasses media as indicated in Table V. Very small amounts of this

TABLE IV

*Treatment of Cuban High Test Molasses with Various Materials*

Treatment	Ingredients added during treatment <sup>†</sup>			Amount treated molasses added to beet molasses medium	Titratable acid as citric
	N/1 H <sub>2</sub> SO <sub>4</sub>	K <sub>4</sub> Fe(CN) <sub>6</sub> ·4H <sub>2</sub> O	Others		
	ml./l.	g/l	g./l.	ml./l.	per cent
A	0	0	—	0	96
				100	35
				200	30
B	8	0	Filter cell, 12	100	42
				200	33
C	8	0	Filter cell, 12	100	30
D	20	0	Filter cell, 20	100	57
				200	38
E	25	0	Filter cell, 20	100	38
				200	34
F	12	0	Norit A, 10	100	43
G	8	0.4	Norit A, 10	100	84
				200	72
				1000	24
H	8	0.8	Norit A, 10	100	73
				200	54
				1000	27
I	8	1.0	Norit A, 10	100	83
				200	85
				1000	26
J	2.2	1.2	Norit A, 10	100	93
				200	79
				1000	28

<sup>†</sup> Ingredients added to C.H.T. molasses before dilution of solution with distilled water to 1 l. (140 g. sucrose). Final pH adjusted to 6.5 with KOH, and precipitation period of 2 or 3 days at 10°C. allowed before decantation and testing of supernatant solution. The figures given in column 5 indicate the quantities of treated molasses (e.g., 100) added to 1000 ml. of beet molasses medium (e.g., total 1100 ml.).

Organism: *Aspergillus niger* 72-4.

material were effective in reducing the yields of acid. Ash from other samples of cane molasses was also tested with similar results. The ash of untreated beet molasses also had this effect, but the ash from the ferrocyanide treated material did not affect the acid production. A number of metallic salts were also tested but no conclusive results could be obtained, and it seems probable that the effect cannot be ascribed to any one metallic ion, but rather to a mixture.

TABLE V  
Effect of Addition of Molasses Ash to Various Media

Ash added		Medium	Organism	Titratable acid, as citric
Kind	Amount			
	<i>g/l</i>			<i>per cent</i>
Cuban High Test	0	Synthetic	<i>A. niger</i> 62	69
	0.3			48
	0.6			41
	1.3			36
	2.6			30
	3.2			18
	0	Beet molasses	<i>A. niger</i> 72-4	98
	0.03			90
	0.11			90
	0.20			90
	0.30			66
	0.40			36
	0.50			36
Peruvian	0.40	Beet molasses	<i>A. niger</i> 72-4	79
Florida A	0.50	Beet molasses	<i>A. niger</i> 72-4	78
Florida B	0.30	Beet molasses	<i>A. niger</i> 72-4	49
Cuban blackstrap	0.50	Beet molasses	<i>A. niger</i> 72-4	37
Michigan beet	0.03	Beet molasses	<i>A. niger</i> 72-4	50
Treated Michigan beet*	4.0	Beet molasses	<i>A. niger</i> 72-4	95

\* Ferrocyanide treated molasses.

## DISCUSSION

The instability of mold cultures as encountered recently in the penicillin fermentation (10) sets a precedent perhaps for considering the change in culture noted here as a possible example of degeneration. However, it is possible that other factors, such as age of spores or sporulation medium, may be responsible for this change.

From the experiments mentioned above it seems quite logical to conclude that at least some of the inhibitory material present in cane molasses is inorganic in nature. Since the ferrocyanide treatment is effective in removing the inhibitory material from beet molasses, it is not surprising that it is somewhat effective with the cane molasses. This is in agreement with the results reported by Mezzadrolì (6) who also found charcoal to have a favorable effect. Karow (4) reports that

a combination of ion exchange and charcoal treatments was very effective in removing the inhibitory substances for the production of citric acid under submerged conditions.

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### SUMMARY

The inorganic constituents of cane molasses are, at least partially, responsible for the low yields of citric acid obtained when media containing cane molasses is fermented by high citric acid-producing strains of *Aspergillus niger* in surface culture. Pretreatment of the cane molasses with cation exchange materials or by precipitation with potassium ferrocyanide removes some of the inhibitory material and results in higher yields of citric acid.

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# Effect of Metallic Ions on the Production of Citric Acid by *Aspergillus niger*\*

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## INTRODUCTION

The effect of low concentrations of metallic ions on the growth of *Aspergillus niger* has been extensively investigated. The review by Foster (4) summarizes the reports on this subject. However, the existing data on the effects of metallic ions on citric acid production by *A. niger* are somewhat contradictory.

Quilico and di Capua (8) have reported that the addition of 10 drops of a 0.1 *N* FeCl<sub>3</sub> solution per 100 ml. of medium was sufficient to decrease the production of citric acid by one strain of *A. niger* from 14.8 to 0.7 weight-% of the available sugar. With another strain the addition of 6 drops of the FeCl<sub>3</sub> solution increased the citric acid production from 5.6 to 9.3%. Bolcato (1) found that the addition of 0.3 ml. of 0.5 *N* FeCl<sub>3</sub> solution per 100 ml. of medium decreased the yield of citric acid by his strain of *A. niger* from 8.5 to 5.5%. Porges (7) has reported that the addition of 0.02 g. of FeCl<sub>3</sub> to a liter of medium increased the yield of citric acid from 3.2 to 13.0%. The addition of 0.01 g. of NiSO<sub>4</sub>, MnSO<sub>4</sub>, or CuSO<sub>4</sub> to a liter of this medium decreased the quantity of citric acid produced; the addition of ZnSO<sub>4</sub> did not affect the yield appreciably. Chrzaszcz and Peyros (2) have studied the effect of iron, zinc and mixtures of iron and zinc, on citric acid production by four strains of *A. niger*. When "a few drops" of FeCl<sub>3</sub> solution were added to the medium the quantity of citric acid produced by strain W.2 was increased from 44.6 to 84.6% of the available sugar. The addition of 0.002% ZnSO<sub>4</sub> to the basal medium decreased the yield from 44.6 to 37.4%. The addition of a mixture of the ions at these levels gave a yield of 39.2%. The same effect on citric acid production was observed with the other three strains. However, all produced much less citric acid than strain W.2.\*

These reports indicate that the metallic ion content of the medium may markedly affect the production of citric acid by this organism.

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However, with the exception of the results obtained by Chrzaszcz and Peyros (2) with one strain of *A. niger*, the yields of citric acid obtained in these investigations are very low, indicating that the yields were limited by factors other than the metallic ion concentrations. In none of the reports is the metallic ion content of the basal medium stated. In the experiments presented in this report an effort has been made to determine the effects of the addition of known quantities of many metallic ions to a highly purified medium on the citric acid production by several strains of *A. niger*.

Preliminary experiments in this investigation indicated that commercial grades of sugar contained sufficient quantities of metallic ions to decrease the yield of citric acid. These metallic cations were removed by passage of the sugar solutions over a cationic exchange material. This method is effective, convenient and industrially feasible.

## METHODS

Five strains of *A. niger* obtained from the Wisconsin collection were used in the following experiments. *A. niger* 59 originally came from the collection of Dr. K. Bernhauer. *A. niger* 62 and *A. niger* 69 are both presumably the strain that was used in the experiments reported by Wells *et al.* (10). The former was obtained from Dr. P. A. Wells in 1938 and the latter from Dr. K. B. Raper in 1941. *A. niger* 70 was also received from Dr. Raper, and is presumably the culture used by Doelger and Prescott (3). *A. niger* 72 is the *A. niger* 1015 in the American Type Culture Collection.

The mold stocks were carried on soil; sucrose agar slants were inoculated from these stocks for use as fermentation inoculum. A portion of a spore suspension obtained by suspending the spores from an agar slant in sterile water was used to inoculate the fermentation flasks. The age of the spores used for fermentation inoculum varied from 6 to 40 days. In experiments reported elsewhere in detail (6), it was found that this variation in the age of the spores, and also large variations in the size of inoculum, had little or no effect on the fermentation. The fermentations were incubated at 30°C. for 10–12 days. The length of the fermentation periods necessary to reach maximum acid production are given in the tables.

The fermentations were carried out in cotton plugged soft glass rectangular six ounce bottles. Results obtained with these soft glass bottles were comparable to those obtained with fermentations conducted in pyrex Erlenmeyer flasks. Fifty ml. of medium were added to each bottle. This amount gave a 1 cm. layer of medium when the bottles were placed in a horizontal position. In the results reported in the following tables at least three fermentations were harvested at the same time, and most of

the experiments were repeated several times. Since traces of organic and inorganic materials markedly influence the fermentation, it was found necessary to clean the bottles with dichromate cleaning solution, and to rinse them at least six times with water (the last three times with distilled water) to obtain reproducible results and agreement between replicate fermentations.

The media were adjusted to pH 2.3 with HCl before bottling and subsequent autoclaving at 120°C. for 20 minutes. All the sucrose was hydrolyzed by this procedure. The sucrose used in these media was a loaf sucrose produced by the "adant process," by the American Sugar Refining Company, New York. The inorganic salts used in the media were of reagent grade. The following reagent grade salts were used as sources of the indicated metallic elements in the experiments:  $\text{Fe NH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  (Fe);  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (Mn);  $\text{CaCl}_2$  (Ca);  $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$  (Al);  $\text{K}_2\text{Cr}_2\text{O}_7$  (Cr);  $(\text{NH}_4)_2\text{MoO}_7 \cdot 4\text{H}_2\text{O}$  (Mo);  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (Cu);  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (Zn). These metallic ions were added to the basal medium where indicated before sterilization. The composition of the basal media used is given in Table I.

TABLE I  
*Composition of Media*

Component	Medium A	Medium B
	g./l.	g./l.
Sucrose	140	140
$\text{KH}_2\text{PO}_4$	1.00	0
$\text{K}_2\text{HPO}_4$	0	1.00
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.25	0.23
$\text{NH}_4\text{NO}_3$	2.25	2.23
HCl (to pH 2.3)	0.232	0.325
Trace elements*	γ/l.	γ/l.
Manganese	18	16
Copper	62	92
Zinc	5	—
Iron	162	102

\* Determined spectrographically on acid ash of medium.

At the end of the fermentation period, the bottles were steamed for twenty minutes to kill the mold. The contents of the bottles, including mycelium, were diluted to 100 ml. (the bottles were calibrated at this volume). An aliquot was removed and titrated to the phenolphthalein end-point with standard alkali. In some of the following experiments the titratable acidity was calculated as citric acid. In others (Tables III and IV) both oxalic acid and citric acid were determined. The former was estimated by precipitation as the calcium salt in dilute solution at pH 5 and subsequent permanganate oxidation, and the latter by the pentabromoacetone method as described by Perlman, Lardy, and Johnson (5). In the experiments reported in Tables IX and X the oxalic acid was determined by this procedure and the residual acidity was calculated as citric acid.

As may be seen in Table II, these two acids accounted for all the acid produced by these strains under the conditions of these experiments. For routine testing of the effect of added metallic ions on acid production by two strains (No. 62 and No. 72) the total acidity was

TABLE II

*Titrateable Acidity, Citric Acid and Oxalic Acid Produced by Various Strains of A. niger*

Strain	0.1 N NaOH per ml. of fermented medium	0.1 N NaOH equivalent to citric acid formed	0.1 N NaOH equivalent to oxalic acid formed	Difference
	ml.	ml.	ml.	ml.
59	2.48	1.55	0.89	-0.04
62	1.58	1.46	0.10	-0.02
69	2.29	1.61	0.72	+0.04
70	2.49	2.21	0.37	+0.09
72	1.51	1.23	0.30	+0.02

calculated as citric acid (Tables V, VI, VII, and VIII). The oxalic acid formed by strain 62 in these fermentations was never more than 10% of the available sucrose; it approached 25% for strain 72. The yields of acids reported in the tables are calculated thus:

$$\frac{\text{g. citric acid (monohydrate) produced}}{\text{g. sugar available}} \times 100 = \text{percentage yield,}$$

$$\frac{\text{g. oxalic acid (dihydrate) produced}}{\text{g. sugar available}} \times 100 = \text{percentage yield.}$$

If all the carbon in the sucrose were converted to citric acid, a yield of 123% would be possible. This method of calculation is used since the citric acid is recovered from the fermented medium as the monohydrate. All fermentations, except those reported in Table III, were of at least 10 days duration. The maximum yield was usually obtained between the eighth and tenth days of the fermentation.

Fermented sugar was determined by the method of Shaffer and Somogyi (9). For practical purposes the yields of citric acid are calculated on the basis of the sugar available for fermentation. Since the specific action of the added trace elements on the metabolism of the mold was not under investigation, complete carbon analyses of the fermentations were not made. In the experiments summarized in

## CITRIC ACID PRODUCTION

Tables V, VI, VII and VIII, dealing with the routine testing of the effects of many metallic elements on acid production, sugar fermentation was not followed.

## EFFECTS OF ADDED METALLIC IONS ON ACID PRODUCTION

In Table III data showing the effects of added iron on the fermentation of sucrose by strain 62 are presented. Both the iron concentration and the sucrose concentration of the medium were varied in this experiment. The results indicated that sucrose concentrations above 140 g./l. gave reduced yields of citric acid. It appeared that the addi-

TABLE III

*Effect of Added Iron on Acid Formation from Sucrose (Strain 62)*

Sucrose concentration	Iron added per liter											
	0.0 mg.				0.1 mg.				1.0 mg.			
	Sucrose fermented	Yield of citric acid	Yield of oxalic acid	Length of fermentation	Sucrose fermented	Yield of citric acid	Yield of oxalic acid	Length of fermentation	Sucrose fermented	Yield of citric acid	Yield of oxalic acid	Length of fermentation
	g./l.	per cent	per cent	per cent	days	per cent	per cent	per cent	days	per cent	per cent	per cent
100	98.8	49.2	5.4	8	100.0	72.0	4.7	10	98.3	54.1	5.9	10
120	89.0	44.3	6.2	10	95.0	68.0	9.4	10	87.0	52.9	10.8	10
140	80.6	43.9	9.1	10	92.2	67.8	9.3	10	88.0	63.3	8.3	10
160	81.2	40.3	5.3	11	90.0	55.2	4.9	10	85.3	50.1	5.7	11
180	75.5	39.2	6.1	12	91.5	45.8	5.0	12	80.0	40.0	7.1	12
200	75.0	35.1	6.8	14	88.7	40.1	5.1	14	78.1	35.3	6.2	14

Medium A was used. Data are given only for the day on which maximum yield was obtained.

tion of 0.1 mg. of iron to this medium gave optimum results regardless of the original sucrose concentration in the medium.

The results of experiments on the effects of added iron on the fermentations by other strains of *A. niger* are shown in Table IV. Basal medium B was used in these experiments. With strains 62, 69, 70 and 72, the optimum iron concentration for citric acid production was between 0.1 and 1.0 mg./l., while 10 mg./l. seemed optimal for strain 59. Under the conditions of these experiments, best results were obtained with strain 62, while strain 69 did very poorly despite the fact that the two strains are presumably identical.

TABLE IV

*Effect of Iron on Acid Formation by Various Strains of Aspergillus niger*

Strain	Iron added	Yield of citric acid	Yield of oxalic acid	Length of fermentation
	<i>mg./l.</i>	<i>per cent</i>	<i>per cent</i>	<i>days</i>
59	0.00	0.2	4.8	10
	0.01	3.0	7.5	10
	0.10	1.4	8.2	10
	1.00	6.4	7.8	10
	3.00	30.9	19.8	10
	10.00	48.5	16.1	10
62	0.00	17.7	3.9	8
	0.01	44.0	2.3	10
	0.10	61.2	3.9	10
	1.00	58.4	9.1	8
	3.00	32.5	4.0	8
	10.00	33.1	7.0	10
69	0.00	13.1	5.0	10
	0.01	10.3	7.1	10
	0.10	37.4	7.4	10
	1.00	31.5	6.5	10
	3.00	32.8	6.3	10
	10.00	32.9	13.8	10
70	0.00	25.2	21.8	10
	0.01	31.9	26.4	10
	0.10	40.9	10.9	10
	1.00	43.5	10.0	10
	3.00	36.0	10.6	10
	10.00	38.2	8.9	10
72	0.00	18.2	24.0	10
	0.01	28.2	12.4	10
	0.10	26.5	16.0	10
	1.00	23.1	14.0	8
	3.00	24.9	11.0	6
	10.00	19.0	13.5	6

Medium B was used. Yields reported are for day of maximum citric acid production.

Some results of a study of the effects of other metallic ions are given in Table V. As may be seen from this table, aluminum, chromium, iron and manganese were the only metallic ions which were stimulatory to acid production under the conditions of the experiment. Others,

TABLE V

*Effect of Added Metallic Ions on Acid Formation from Sucrose (Strain 62)*

Quantity of metallic ion added	Yield of acid obtained when indicated ion was added							
	Iron	Manganese	Aluminum	Molybdenum <sup>1</sup>	Copper	Zinc	Calcium	Chromium
mg/l.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
0.0	48.2	48.1	51.4	49.4	52.0	47.4	48.3	47.5
0.01		50.5	62.6	53.4	47.5	35.7	50.5	34.5
0.03	57.5	51.9			30.4			
0.1	67.2	68.5	40.4	51.3	29.2	28.3	18.7	54.8
0.3	61.5	66.1			19.0			
1.0	52.4		39.6	49.3	14.4	37.5	23.5	65.4
3.0	50.5	41.2						
10.0	44.8		33.5	48.2		28.5	19.8	51.5
30.0	42.9							
100.0			2.9	42.1		21.0	33.3	

Medium A was used. Yields of citric acid are calculated from titratable acidity data taken at the time of maximum yield. This occurred after the following incubation periods: iron, 10 days; manganese, 8 days; aluminum, 8 days; molybdenum, 10 days; zinc, 10 days; chromium, 10 days.

<sup>1</sup> The amounts are given in  $\gamma$  instead of mg.

such as zinc, were inhibitory at all concentrations tested. When other stimulatory elements were used in combination with iron, the yields obtained were, in general, no better than those obtained with iron alone. Data obtained on medium A are given in Table VI. However, in one series of experiments made with medium B, combinations of iron and manganese gave better yields than either element alone. The data obtained are summarized in Table VII.

The optimum metallic ion concentration for stimulation of citric acid production seems to vary with the strain of *A. niger*. In Table VIII data collected on the effect of the addition of iron, manganese and molybdenum on the conversion of sucrose to citric acid by strain 72 are presented. Molybdenum was definitely stimulatory for this strain, while it had little effect on strain 62 (Table V). Iron, however, had



TABLE VI

*Effect of Addition of Mixtures of Iron and Other Metallic Ions on  
Acid Formation from Sucrose (Strain 62)*

## A. Iron and Aluminum

Iron added	Aluminum added	Yield of citric acid	Length of fermentation
<i>mg./l.</i>	<i>mg./l.</i>	<i>per cent</i>	<i>days</i>
0.05	0.0	64.5	8
0.05	0.005	62.5	8
0.05	0.01	54.2	9
0.05	0.05	46.1	9
0.1	0.005	61.5	10
0.1	0.01	53.8	8
0.1	0.05	51.0	9

## B. Iron and Manganese

Iron added	Manganese added	Yield of citric acid	Length of fermentation
<i>mg./l.</i>	<i>mg./l.</i>	<i>per cent</i>	<i>days</i>
0.05	0.0	61.5	10
0.05	0.1	24.3	10
0.1	0.01	39.5	8
0.1	0.03	34.5	8
0.1	0.1	30.8	9
0.1	0.3	34.4	10
0.3	0.1	46.5	8

## C. Iron and Chromium

Iron added	Chromium added	Yield of citric acid	Length of fermentation
<i>mg./l.</i>	<i>mg./l.</i>	<i>per cent</i>	<i>days</i>
0.05	0.0	63.8	8
0.05	1.0	61.1	9
0.1	0.1	63.4	8
0.1	0.5	64.9	8
0.1	1.0	58.1	10
0.5	1.0	61.5	8
1.0	1.0	58.6	8

Medium A was used. Titratable acidity was calculated as citric acid on day of maximum acidity.

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TABLE VII

*Effect of Addition of Mixtures of Iron and Manganese on  
Acid Formation from Sucrose (Strain 62)*

Iron added	Manganese added	Yield of citric acid	Length fermentation
<i>mg./l.</i>	<i>mg./l.</i>	<i>per cent</i>	<i>days</i>
0.0	0.0	35.3	8
0.1	0.0	45.0	8
0.3	0.0	49.0	9
1.0	0.0	58.2	8
3.0	0.0	53.0	8
10.0	0.0	53.3	8
0.0	0.001	46.0	9
0.0	0.003	58.3	9
0.0	0.01	54.2	9
0.0	0.03	41.7	8
0.0	0.1	30.3	10
0.1	0.001	53.3	8
0.1	0.003	64.1	9
0.1	0.01	59.1	8
0.1	0.03	46.5	8
0.1	0.1	43.3	9
0.3	0.001	62.3	8
0.3	0.003	61.3	8
0.3	0.01	44.2	8
0.3	0.03	36.8	9
0.3	0.1	43.3	9
1.0	0.001	59.0	8
1.0	0.003	64.0	8
1.0	0.01	43.5	8
1.0	0.03	36.8	8
1.0	0.1	39.1	9
3.0	0.001	58.7	8
3.0	0.003	69.2	8
3.0	0.01	44.7	8
3.0	0.03	46.2	8
3.0	0.1	40.7	8
10.0	0.001	62.3	8
10.0	0.003	59.7	8
10.0	0.01	64.7	8
10.0	0.03	51.7	10
10.0	0.1	41.0	9

Medium B was used. Titratable acidity calculated as citric acid on day of maximum acidity.

TABLE VIII

*Effect of Added Metallic Ions on Acid Production (Strain 72)*

Quantity of metallic ion added	Yield of acid obtained when indicated ion was added		
	Molybdenum	Manganese	Iron
$\gamma/l.$	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0	55.6*	52.5	55.6
1	70.5	50.6	
5	66.3	42.1	
10	48.6	24.5	
50	49.5	25.3	47.5
100		22.5	48.5
500			49.3
1000			51.6
5000			44.4
10000			37.5

Medium A was used. Maximum titratable acidity calculated as citric acid. The maximum acidity was reached after the following incubation periods: molybdenum, 8 days; manganese, 10 days; iron, 10 days.

\* This sample analyzed: 41.5% citric acid; 13% oxalic acid (calculated on available sugar).

little effect, and was somewhat inhibitory for strain 72, while it was definitely stimulatory for strain 62. Manganese was inhibitory for strain 72 at a very low level.

#### ACID PRODUCTION FROM GLUCOSE AND COMMERCIAL SUGAR

It is probable that the results obtained with sucrose are typical of those to be obtained with other sugars under similar conditions. The results obtained when varying amounts of iron were added to the basal medium in which C. P. glucose was substituted for the sucrose, are found in Table IX. Here, 0.1 mg./l. of iron seemed optimal.

Less pure grades of commercial sucrose and commercial glucose were tested as substitutes for the pure grade used in the above mentioned experiments. Media prepared with these commercial grades of sugars gave very low yields of citric acid. It was thought that these sugars were contaminated with significant amounts of metallic substances (the cane sucrose contained 1.5 mg. of manganese/100 g.), and they were therefore treated by passage over cationic exchange materials.

TABLE IX

*Effect of Iron on Acid Formation from Glucose (Strain 62)*

Iron added	Yield of citric acid	Yield of oxalic acid	Glucose fermented	Length of fermentation
mg./l.	per cent	per cent	per cent	days
0	42.5	6.0	85.5	10
0.1	65.4	8.8	98.8	9
1.0	50.3	7.9	80.7	11

Salts as indicated in medium A were used. Yields reported are obtained on day of maximum citric acid production.

The resulting solutions after addition of the usual salts, when fermented by strain 62, gave good yields of citric acid, as is shown in Table X.

A 28% sugar solution was passed over the cationic exchange material at a rate of 1 l/hr. Before passage the pH of the solution was approximately 6.0 while, after treatment, the pH was about 4.8. The bed was approximately 12 inches in length and 1 inch in diameter. The cationic exchange material used in these experiments (Table X) was "Alkalex," a product of Research Products Corporation, Madison, Wisconsin. This material was operated on a hydrogen cycle (sulfuric acid). After treatment, the solution was diluted to 14% sugar, the in

TABLE X

*Effect of Treatment of Commercial Sugars by Ionic Exchange Method on Acid Formation (Strain 62)*

Sugar	Treatment	Yield of citric acid	Yield of oxalic acid	Sugar fermented	Length of fermentation
		per cent	per cent	per cent	days
Cane Sucrose	None	21.4*		34.4	8
	Cationic exchange	64.0	4.1	98.0	8
Beet Sucrose	None	11.3*		17.6	8
	Cationic exchange	66.8	5.0	97.5	10
Glucose†	None	20.5*		33.2	8
	Cationic exchange	65.0	7.2	99.0	10

Salts indicated in medium A were used. Yields reported are for day of maximum yield of citric acid.

\* Titratable acidity calculated as citric acid.

† "Cerelease" (glucose monohydrate).

organic salts, including 0.1 mg./l. of iron, added, and the resulting medium bottled, sterilized and inoculated, as in the previous experiments. Other cationic exchange materials which were tested and gave comparable results were "Amberlite IR-100" from Resinous Products and Chemical Company, Philadelphia, and "Zeo-Karb H" from the Permutit Co., New York. Several anion exchange materials were tested but had little effect on the fermentation. Evidently the inhibitory material present in these commercial sugars is cationic in character. When the treated material was passed over the anion exchange material and then for a second time over the cationic exchange material no better citric acid production was obtained. Probably a sufficient amount of the contaminating substance was removed by the initial treatment with one cationic exchange material. Cationic exchange treatment appears to be a convenient method for reducing the metallic ion content of microbiological media.

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#### SUMMARY

The addition of 0.1 mg. of iron, 0.1 mg. of manganese, 0.1 mg. of aluminum, or 1.0 mg. of chromium, to a liter of a sucrose-salts medium increased the conversion of sucrose to citric acid by a strain of *Aspergillus niger* by 50% (45 to 67% of the available sucrose). Mixtures of these metallic ions at these or lower levels were no better than the single elements in increasing the conversion of sucrose to citric acid on media made with water from one still. On another medium, combinations of iron and manganese were better than either alone.

The optimum concentration of stimulatory metallic elements varied from one strain of *A. niger* to another on the same medium. Metallic ions stimulatory for one strain may have no effect on another strain under the same conditions.

A cationic exchange treatment of commercial sugars increased the conversion of available sugar to citric acid more than three-fold (20-65% of the available sugar).

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# Lipolytic Activities of Anaerobic Bacteria\*

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## INTRODUCTION

Although many investigators have stressed the aerobic nature of fat decomposition by microorganisms, evidence is accumulating to show that lipids may be actively consumed by anaerobes. While it has been indicated that anaerobic utilization occurs with difficulty (1-5), the opinion that oxygen is essential to bacterial lipolysis<sup>1</sup> has been invalidated by repeated demonstrations of hydrolytic activity in an anaerobic environment (6-24). Further evidence suggesting lipid cleavage in the absence of oxygen is noted in the marked activation of various non-microbial esterases and lipases by reducing agents (25-28).

The scarcity of comprehensive investigations into the anaerobic consumption of lipids, together with the possible role of fatty acids as precursors to hydrocarbon formation (29, 30), prompted the initiation of such studies in this laboratory. Logically, the question of lipolysis by anaerobic bacteria is fundamental to this type of experimentation.

## METHODS

Enrichment techniques were selected as the most likely means of demonstrating lipolytic anaerobes. The procedure adopted consisted of establishing anaerobic growth in the presence of a lipid substrate, followed by the application of tests diagnostic for the presence of lipoclasts.

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<sup>1</sup> The term *lipolysis* can indicate various molecular disruptions involving lipids. The words *lipolysis*, *lipoclasia* and their adjectival derivatives are employed in the ensuing discussion to signify the hydrolytic cleavage of an ester into its alcoholic and fatty-acid components.



Both organic and inorganic forms of nitrogen have been shown to suffice for the nutrition of aerobic lipoclasts (2, 8, 31). To increase the likelihood of isolations, enrichment cultures were customarily prepared in two series. One of these received ammonium sulfate plus peptone and the other the inorganic salt alone. Nitrate was not employed because of the large and varied aerobic population capable of activating it as an hydrogen acceptor and thereby growing in a pseudo-anaerobic manner.

The mineral salts solution with which the several enrichment media were prepared was composed of:

$K_2HPO_4$ .....	1.0 g.
$(NH_4)_2SO_4$ .....	1.0 g.
$MgSO_4 \cdot 7H_2O$ ..	0.2 g.
$FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ .....	0.1 g.
Water.....	1000.0 ml.

Both fresh water and aged sea water (32) were used, the type being determined by the salinity requirements of the microorganisms. Cultures of marine bacteria developed satisfactorily in solutions containing equal volumes of distilled water and sea water. The development of non-marine cultures was enhanced by the addition of 5% of aged sea water to the media.

Peptone, when added, was employed in a concentration of 0.5%. Lipids were added in amounts ranging from 0.1 to 0.5%, soluble compounds being added directly to the medium before autoclaving. Insoluble substrates were thoroughly emulsified and were sterilized separately. Such emulsions normally contained:

Gum arabic.....	0.4 g.
Neutral red base.....	0.04 g.
Lipid.....	10.0 ml.
Distilled water (pH 7.8).....	50.0 ml.

The preparation of neutral red base and its function as an indicator of lipolysis have been discussed by Knaysi (33, 34). The dye was dissolved in the lipid, the remaining constituents then added and the whole mixed by agitation in a mechanical blender. Emulsification was completed by repeated passage of the mixture through a hand-operated homogenizer. The addition of gum arabic assured emulsion stability during autoclaving and greatly aided the fine dispersion of lipid droplets. The emulsion was used in a proportion of 30 ml./l. of medium. Water-insoluble solids, such as tripalmitin, were prepared by shaking an alcohol-ether solution of the compound with water. Subsequent evaporation of the volatile solvent produced a finely dispersed emulsion, the lipid content of which was below 1%.

All preparations were autoclaved for 20 minutes at 124°C. The sterilized media were adjusted to pH 7.5 with sterile HCl or NaOH, and autoclaved emulsions of lipids undergoing investigation were then added.

Cultures were prepared in 60 ml. glass-stoppered bottles. Although the mixed flora of the inoculum normally succeeded in lowering the *O/R* potential to a level compatible with the growth of anaerobes, proper poisoning of the media was often an advantage. For this reason, 0.02% of ascorbic acid (35) was generally added to the mineral salts solution before sterilization.

Inocula were derived from both sedimentary and fluid materials. In the case of

the former, the solids were suspended in sterile water and the supernatant liquid used to seed the medium. Inoculated bottles were incubated in the dark for periods of at least 30 days at 27°C. The development of turbidity greater than that of uninoculated controls was considered as evidence of growth. Emulsified substrates masked this turbidity, but a change in indicator color revealed bacterial development. In doubtful instances cultures were plated.

Cultures developing under such enrichment conditions were then tested for their content of anaerobic lipoclasts in a medium containing the mineral salts solution and the following:

Agar.....	2.0%
Peptone (when added).....	0.5%
Ascorbic acid.....	0.02%

Lipid emulsions similar to those already described were added after sterilization. The dye content of these preparations was increased from 0.04 to 0.06 g./10 ml. of lipid. Insoluble solid lipids were emulsified by shaking with the agar medium at temperatures above the melting point of the substrate. Since these temperatures were usually inimical to the bacteria, the use of such substrates necessitated the preparation of streak plates. Except for these cases, pour plates were employed.

Plates were incubated in hydrogen jars of the McIntosh-Fildes type at 27°C. The period of incubation varied from 7 to 14 days, occasionally extending to longer intervals. At pH 7.5, plates of lipid agar containing neutral red base were amber in color. Lipolytic colonies were often surrounded by a red areola, while the colonies themselves were extremely dark red. Non-lipolytic bacteria did not produce this color change. No growth inhibition by the dye was detected.

### RESULTS WITH ENRICHMENT SUBSTRATES

Original cultures were prepared on several lipid substrates. The anaerobic character of this development was confirmed by electro-metric measurements of the *O/R* potential. The majority of cultures demonstrated  $E_h$  values between -100 and -200 millivolts, with some reaching more negative levels.

The cultures produced by these methods were then tested for their abilities to split tributyrin. Table I outlines the quantitative distribution of such lipoclasts in cultures derived from the several substrates tested, and Table II apportions the lipolytic anaerobes among the various types of materials employed as inocula.

Several cultures, originally produced in butyl lactate solutions, were employed in experiments testing the effect of peptone upon the determination of lipolysis. Cultures produced in the presence of the ester alone or in combination with peptone were plated in tributyrin agar. One series of plates was prepared with ammonium nitrogen, while the other was further enriched with peptone. Of 13 cultures tested, 11

grew and produced visible hydrolysis of tributyrin in the absence of peptone. When peptone was added, 12 cultures developed, but lipolysis was evident in only 7 cases. The criterion of lipoclasia was reaction with neutral red base. Additional investigation revealed this decrease in lipolytic manifestation to be due essentially to a masking of the color change by the alkaline products of peptone decomposition. Occasionally, weakly lipolytic bacteria demonstrated the preferential

TABLE I  
*Anaerobic Lipoclasts Isolated from Cultures Developed by Enrichment  
with Various Lipid Compounds*

Substrate	Source materials employed as inocula	Cultures growing in presence of lipid substrate	Growing cultures which hydrolyzed tributyrin
Butyl formate	25	1	0
Ethyl acetate	16	5	3
Amyl acetate	16	1	0
Monoacetin	89	8	0
Triacetin	7	5	0
Ethyl propionate	11	0	—
Ethyl propionate plus peptone	11	7	6
Tripropionin	55	0	—
Tributyrin	31	19	5
Tributyrin plus peptone	31	19	6
Tripalmitin	55	0	—
Butyl stearate	25	1	0
Butyl lactate	45	17	2
Butyl lactate plus peptone	45	33	10
Calcium lactate	41*	41	26
Cholesterol	24	0	—

\* This was a group of mixed cultures originally developed on calcium lactate and containing sulfate-reducing anaerobes.

consumption of peptone, but vigorous lipoclasts hydrolyzed lipids while also utilizing peptone. Lipolysis was then recognized in the formation of extra-colonial clearing zones which signified the dissolution of the lipid substrate. Attempts were made to obviate the difficulty of neutral red base competition with the alkaline products of peptone transformation for liberated fatty acids by increasing the buffer content of the medium. However, concentrations of  $K_2HPO_4$  as high as 0.5% failed to permit reddening when peptone was consumed.

## HYDROLYSES OF ESTERS CONTAINING INSOLUBLE FATTY ACIDS

A series of esters and allied substances was next subjected to the attack of vigorously lipolytic anaerobes. Peptone was not employed in the customary plate cultures, which were incubated for periods ranging from 14 to 20 days at 27°C. Results are depicted in Table III.

The organisms used in this study were originally isolated on tributyrin. The data exemplify a somewhat inconsistent but overall

TABLE II

*The Distribution of Anaerobic Lipoclasts in Naturally Occurring Materials*

Type of inoculum	Samples tested	Number of cultures developed	
		Total	Lipolytic
Crude oil	55	7	1
Oil-well brine	66	15	8
Petroleum storage water	5	5	5
Oil-well core	3	0	—
Oil sand	8	1	1
Asphalt	12	0	—
Paraffin earth <sup>*</sup>	5	3	3
Saline waste water <sup>†</sup>	15	11	8
Saline lake water	5	5	1
Marine mud	53	48	27
Sea water (aquarium)	2	2	2
Fresh-water estuary	3	2	2

\* Waxy dirt occurring in surface and subsurface layers often associated with gas seepage in regions of petroleum formations.

† Water containing relatively high concentrations of dissolved salts and emerging as a by-product of chemical manufactures.

reduction in lipolytic display as higher or more refractory esters were tested. While these findings are not intended to bear upon the controversial distinctions between esterase and lipase activities, the hydrolyses of esters containing long-chain fatty acids by tributyrin-splitting anaerobes do point to generalized lipolytic powers. It may, perhaps, be postulated that hydrolytic failure here is due to the physical and chemical resistance of the substrate rather than to the inability of an organism to produce a specific enzyme.

TABLE III  
*Hydrolyses of Esters Containing Insoluble Fatty Acids by  
 Lipoclasts under Anaerobic Conditions*

Substrate	Cultures tested	Cultures lipolytic
Tricaprylin	6	6
Ethyl laurate	6	6
Trilaurin	8	7
Trimyristin	8	6
Tripalmitin	8	7
Methyl stearate	6	5
Ethyl stearate	6	6
Butyl stearate	8	8
iso-Amyl stearate	6	6
Phenyl stearate	8	5
Tristearin	8	4
Triolein	6	6
Coconut oil	8	3
Corn oil	8	3
Cottonseed oil	8	3
Olive oil	8	3
Palm oil	8	4
Sperm oil	8	3

#### LIPOLYTIC ACTIVITY OF SULFATE REDUCERS

In addition to the various lipolytic bacteria detected by enrichment procedures, a series of experiments was devised to test the lipid-hydrolyzing tendencies of typical sulfate-reducing anaerobes. The occurrence of these bacteria in marine bottom deposits (36) indicated the need for such study as a means of determining additional incitants in the preliminary stages of anaerobic lipid transformation.

The organisms used in the investigation were obtained largely from a series of mixed cultures which had been developed previously in mineral salts solutions enriched with lactate. Many of the cultures contained anaerobic lipoclasts (see Table I), a large number of which showed no similarity to sulfate reducers. Several platings on tributyrin agar were required to develop pure sulfate-reducing cultures which were presumptive lipoclasts. Confirmation of lipolytic powers was attempted by the use of washed cells. Culture C-1P-3, derived from marine mud, was grown on tributyrin agar containing peptone. The cellular crop was harvested by centrifugation, washed once with sea

water and used in heavy sea-water suspension as a lipase source. Screw-capped tubes, each containing 2.0 ml. of cell suspension and 3.0 ml. of 0.5% unbuffered tributyrin emulsion prepared without a stabilizer, were incubated at 27°C. A boiled cell suspension functioned as a control. Hydrolysis of the tributyrin was indicated by a decrease in pH. Table IV illustrates the progress of lipolysis during an interval of 24 hours. Similar results were obtained with cells of culture 388, isolated from a fresh-water estuary, which produced a decrease in pH of 1.61 for the viable cells and of 0.27 for the control. The presence of oxygen did not materially affect the action of these cells upon tributyrin under the conditions described. Duplicate preparations incubated in evacu-

TABLE IV

*Changes in pH Resulting from the Hydrolysis of Unbuffered Tributyrin by Washed Cells of Anaerobic Sulfate-Reducing Lipoclast C-1P-3 During Incubation for 24 Hours at 27°C.*

Hours of incubation	pH	
	Viable cells	Boiled cells
0	8.05	8.05
1	6.13	7.90
2	5.78	7.80
3	5.64	7.64
4.5	5.50	7.65
20	4.98	7.41
24	4.93	7.40

ated Thunberg tubes produced pH changes very similar to those recorded in Table IV. This observation agrees with that of Fleming and Neill (21) who reported that the lipase of the anaerobe *Clostridium perfringens* was unaffected in its tributyrin-splitting activity by the presence of air and by a 0.1 M concentration of H<sub>2</sub>O<sub>2</sub>.

Although washed cells of the sulfate reducer were functional lipolytically in an aerobic environment, cultures of such bacteria are obligately anaerobic. Some appreciation of the reducing potential attained by lipolytic sulfate reducers can be gained from the fact that the cultures readily decolorized neutral red completely when growing on tributyrin in both plate and fluid preparations. It is estimated that the potential approximated -400 millivolts or may even have been

more strongly reducing. Hewitt (37) places the  $E_0$  value of neutral red at  $-325$  millivolts.

### DISCUSSION

The distribution of anaerobic lipoclasts in nature appears to be quite general. Although crude oils were poor sources of the bacteria, lipoclastic cultures were readily developed from brines, storage waters and other materials associated with petroleum products. The organisms were also very abundant in saline mud and water samples. They were present in fresh-water sources, but the extent of their distribution in these environments was not thoroughly investigated.

Colorimetric detection of lipase action in cultures which were stimulated by peptone was rendered difficult by the accumulation of alkaline transformation products which tended to obscure the combination of liberated fatty acid with indicator. It was evident, nevertheless, that peptone did not truly suppress lipase elaboration, for hydrolytic activity was normally recognized in the visible dissolution of the test substrate.

The range of lipids subject to anaerobic hydrolysis is sufficiently great and includes compounds of such molecular weight that true lipase function is evident. No evidence has been accumulated to distinguish between esterase and lipase activity of anaerobic bacteria, but little doubt of lipase elaboration by the organisms can exist.

It has been doubted that anaerobes can efficiently utilize fats as sources of energy (4). However, anaerobic lipolysis in a cultural system would not appear to be restricted unduly by the free-energy changes concerned. There are comparatively few published values for the free energies of formation of esters, but, using data presented by Bull (38), Getman and Daniels (39) and Tausson (40), it is possible to calculate the energetic requirements for the hydrolysis of a few compounds:

1. Ethyl acetate,  $\Delta F^\circ_{298} = -540$  cal.
2. Ethyl butyrate,  $\Delta F^\circ_{298} = 860$  cal.
3. Amyl butyrate,  $\Delta F^\circ_{298} = 860$  cal.
4. Tripalmitin,  $\Delta F^\circ_{298} = 780$  cal.

Although three of the reactions are endothermic, the energy required for the hydrolysis of each substrate is small and could possibly be satisfied by a variety of dehydrogenations. In any event, the positive

free-energy changes do not appear as formidable barriers to lipolysis. This is particularly true with respect to hydrolysis in the presence of the several reacting systems present in the simplest of cultural environments. The demonstrated lipolyses by washed cells may have been motivated by an endogenous respiration which permitted the successful traversing of a positive free-energy gradient.

The range of  $O/R$  potentials over which lipolysis was demonstrated fell within the sphere of anaerobic activity. The distribution of electrode potential values approximated  $-100$  to  $-400$  millivolts, the more negative  $E_h$  figures being characteristic of lipolysis by the obligately anaerobic sulfate reducers.

### SUMMARY

The existence of bacteria capable of anaerobic lipolysis has been confirmed. Among these organisms are the important group of sulfate reducers.

Hydrolyzable substrates have included esters of monohydric alcohols, glycerides of both soluble and insoluble fatty acids and more complex fats and oils.

Peptone consumption by lipolytic anaerobes interfered with the colorimetric detection of lipolysis but only rarely exerted an actual lipid-sparing effect.

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# Glutamic Acid Decarboxylase of Higher Plants

## II. pH-Activity Curve, Reaction Kinetics, Inhibition by Hydroxylamine

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### INTRODUCTION

Okunuki (1) found an enzyme in higher plants which specifically decarboxylated glutamic acid. As attempts to obtain solutions of glutamic acid decarboxylase were unsuccessful, Okunuki concluded that the enzyme was bound to cellular structures.

In a recent report from this laboratory (2, 3) it was shown that clear solutions of glutamic acid decarboxylase can be obtained without difficulty and in good yields from carrots. The decarboxylase activity of 34 plants toward glutamic acid was measured. It was furthermore shown that glutamic acid decarboxylase can be inactivated by dialysis and that activity is restored on addition of pyridoxal phosphate. The conclusion was drawn that glutamic acid decarboxylase of higher plants is apparently a pyridoxal-phosphate-protein complex.

The present communication describes the:

- (1) influence of pH on decarboxylase activity,
- (2) reaction order,
- (3) determination of initial reaction velocities,
- (4) determination of the Michaelis-Menten constant of glutamic acid decarboxylase from carrots, and
- (5) inhibition of the enzyme by hydroxylamine.

### EXPERIMENTAL RESULTS AND DISCUSSION

#### *1. pH-Activity Curve*

Clear carrot extracts were prepared as described (3), 1 ml. phosphate buffer *m*/15, pH 6.4, being used for each gram of fresh tissue. Adjustment of the pH of the enzyme

solution to the desired point was made by adding to 10 ml. of extract an equal volume of *m/15* phosphate buffer mixtures, containing  $K_2HPO_4$ ,  $KH_2PO_4$ , or  $H_3PO_4$  in various proportions. To a 4 ml. sample was added 0.5 ml. of a neutral 1% glutamic acid solution and the pH of the mixture was measured with a glass electrode. The addition of glutamic acid caused a slight shift of the pH toward alkaline (0.03–0.14 pH units) and, during 60 min. of incubation while glutamic acid was decarboxylated, a partial reversal of this shift (0.01–0.04 pH units) took place. A second 4 ml. sample of the enzyme and buffer mixture was used for the determination of enzymatic activity, which was carried out as previously described (3). It was found that the enzymatic activity, as judged by the amount of  $CO_2$  formed in 60 min., remained constant from pH 5.30 to 5.90.

In Fig. 1 activities outside this range are expressed as *per cent* of optimal activity. For the determination of each point outside the optimal range, at least one experiment was performed simultaneously

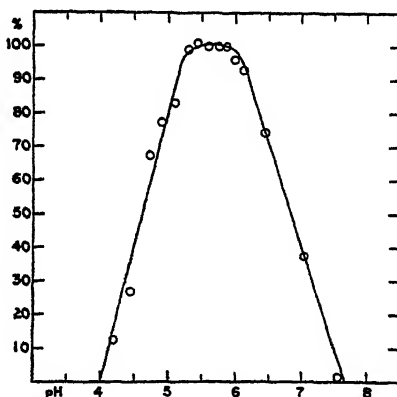


FIG. 1

Influence of pH on the activity of Glutamic Acid Decarboxylase from Carrots

at a pH of 5.60 to 5.80. This procedure was considered necessary to correct for losses in activity which occurred during storage of the extracts. Solutions more acid than pH 4.0 and more alkaline than pH 7.5 showed no activity.

Okunuki (1), using tissue dry powders containing "insoluble" glutamic acid decarboxylase, observed optimal activity near pH 6.0, a slow decrease toward more acid reactions (87% of optimal activity at pH 4.0) and a rapid fall if neutrality or alkaline ranges were approached (47% at pH 7.0, 34% at pH 8).

## 2. Order of Reaction

Abbreviations:  $a$  = amount of substrate present at time zero.

$t$  = reaction period in minutes.

$x$  = amount of substrate decarboxylated at time  $t$  (expressed in mm.<sup>3</sup> CO<sub>2</sub>).

$a - x$  = amount of substrate not yet decarboxylated at time  $t$ .

The time-activity curves for the action of glutamic acid decarboxylase do not represent curves for a monomolecular reaction, as can be easily ascertained by the failure to obtain a straight line by plotting  $\log (a - x)$  against  $t$ . The value for

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

is, accordingly, not found to be a constant but to decrease rapidly while the experiment is in progress, as shown in Table I.

TABLE I

*Time-Activity Data for Glutamic Acid Decarboxylase, Showing Deviation from First Order Reaction*

(Extract I-69, stored 19 hr.,  $a = 5$  mg. glutamic acid or 762 mm.<sup>3</sup> CO<sub>2</sub>)

$t$ (min.)	(mm. <sup>3</sup> CO <sub>2</sub> )	$\log \frac{a}{a-x}$	$k \times 10^3$
10	53	0.0314	3.14
20	95	0.0579	2.90
30	125	0.0779	2.66
40	145	0.0917	2.29
50	159	0.1017	2.03
60	169	0.1089	1.82
70	178	0.1156	1.65
80	182	0.1186	1.48
90	186	0.1216	1.35

When the activity of dialyzed extracts was restored by the addition of pyridoxal phosphate, it was noted that the time-activity curves of such mixtures showed considerably less "die-away" tendencies than the curves of corresponding extracts which were untreated (3). The course of the reaction in the presence of pyridoxal phosphate fits the equation for a first order reaction very well, as shown in Table II.

(The curves labeled PD in the previous report are likewise first order curves. For example  $k \times 10^3$  for the 64 hr. curve is  $4.00 \pm 0.11$  and for the 88 hr. curve  $1.12 \pm 0.04$ .) It is difficult to explain this effect of pyridoxal phosphate. If one recalls that the decarboxylation of glutamic acid leads to the accumulation of  $\gamma$ -aminobutyric acid, one is inclined to assume that this reaction product might function as an inhibitor by reacting with the aldehyde group of the enzyme molecule. Consequently, as the concentration of  $\gamma$ -aminobutyric acid increases, one would expect to observe a progressive decrease in the rate of  $\text{CO}_2$  formation beyond that due to a decrease in substrate concentration. The addition of an excess of pyridoxal phosphate could nullify the

TABLE II

*Monomolecular Reaction Course Data Obtained on addition of Pyridoxal Phosphate*  
(Extract I-69, dialyzed 19 hr., 40  $\gamma$  pyridoxal phosphate added,  
 $\alpha = 5$  mg. glutamic acid or 762 mm.<sup>3</sup>  $\text{CO}_2$ )

$t$ (min.)	(mm. <sup>3</sup> $\text{CO}_2$ )	$\log \frac{\alpha}{\alpha - x}$	$k \times 10^3$
10	50	0.0295	2.95
20	90	0.0545	2.73
30	133	0.0833	2.78
40	170	0.1096	2.74
50	208	0.1385	2.77
60	239	0.1637	2.73
70	271	0.1911	2.73
80	303	0.2202	2.75
90	328	0.2445	2.72

inhibitory effect of  $\gamma$ -aminobutyric acid by binding it through a chemical reaction between the aldehyde group of pyridoxal phosphate and the amino group of the acid. However, this mechanism becomes unlikely in the experiments reported here, if one considers the quantities involved. The amount of pyridoxal phosphate added to the reaction mixture I-69 in Table II was obtained from 80  $\gamma$  barium salt and contained about 40  $\gamma$  or  $0.17 \times 10^{-6}$  mol of pyridoxal phosphate (4). During 90 min. of reaction 328 mm.<sup>3</sup>  $\text{CO}_2$  or  $14.6 \times 10^{-6}$  mol were formed and consequently  $14.6 \times 10^{-6}$  mol of  $\gamma$ -aminobutyric acid have accumulated in the reaction vessel. The total amount of pyridoxal phosphate in the mixture could, therefore, combine with only about 1.2% of the inhibitor and this could not account for the profound change in

the time-activity curve. Pyridoxal, as shown in Table V of the previous communication (3), also restored the activity of dialyzed extracts. In contrast to the effect of pyridoxal phosphate, this did not lead to a first order reaction curve despite the fact that about  $25 \times 10^{-6}$  mol of the aldehyde had been added. The  $k$  values calculated with the equation for a monomolecular reaction were nearly the same and decreased with equal rapidity when the data for the stored extract were compared with those obtained with the dialyzed extract after addition of pyridoxal.

The mechanism by which pyridoxal phosphate changes the reaction course (so that a monomolecular constant is obtained) remains subject to speculation until further experimental evidence permits definite conclusions. Deviation from a first order reaction is probably due to progressive inactivation of the enzyme. This inactivation could come about in two different ways. One possibility is the competitive inhibition by  $\gamma$ -aminobutyric acid. As already pointed out, because of the small amount present, pyridoxal phosphate could not overcome this inhibition by a stoichiometric reaction with the inhibitor. It is possible, however, that the addition of pyridoxal phosphate activates not only glutamic acid decarboxylase but also an enzyme which uses  $\gamma$ -aminobutyric acid as a substrate for deamination-, transamination- or other reactions. This would prevent accumulation of the inhibitor. A second alternative which awaits experimental examination is the possibility that pyridoxal phosphate, when serving as the prosthetic group of glutamic acid decarboxylase, suffers rapid and irreversible damage during the enzymatic reaction. Free pyridoxal phosphate present in excess could then supply active prosthetic groups in exchange for those "deteriorated." This would keep the amount of active enzyme constant until all free pyridoxal phosphate has been utilized.

### *3. Initial Reaction Velocity*

Determination of the velocity of an enzymatic reaction is important for the characterization of an enzymatic system. Knowledge of the reaction velocity of a given preparation not only serves as a numerical expression for its "strength" or enzymatic activity but also makes it possible to express distinctly the effect of preparative procedures and of changes in the physico-chemical environment.

The enzymatic decarboxylation of glutamic acid by carrot extracts follows a first order equation only if pyridoxal phosphate is added in

excess, as shown in Table II. In the absence of pyridoxal phosphate a time-activity curve is obtained which is characterized by a rapid decrease in reaction velocity with time. Obviously, then, it would be desirable to know the maximal reaction velocity, which is the rate of reaction at the time zero, when inactivation of the enzyme has not yet taken place. A simple way to determine initial velocities is the

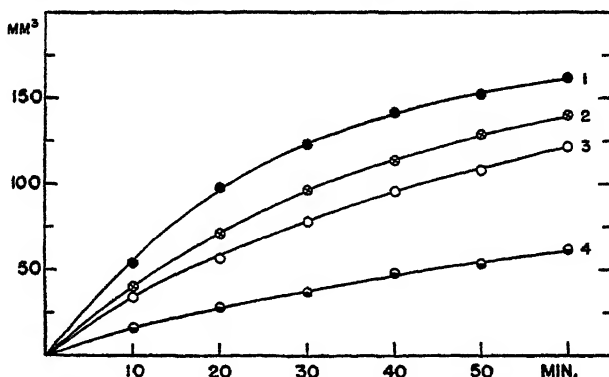


FIG. 2

Time-Activity Curves, Showing the Effect of Hydroxylamine on Glutamic Acid Decarboxylase from Carrots at pH 5.70  
Inhibitor concentrations: zero (curve 1);  $3 \times 10^{-5}$  M/l. (curve 2);  $6 \times 10^{-5}$  M/l. (curve 3);  $10^{-4}$  M/l. (curve 4)

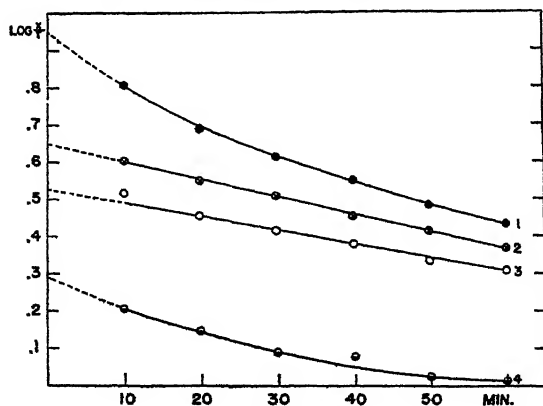


FIG. 3

Graphical Determination of Initial Velocities by Extrapolating  $\log x/t$  for Time Zero  
Numbers correspond to curves of Fig. 2.

graphical extrapolation method. The procedure consists in plotting observed velocities  $x/t$  against the corresponding values of  $t$  and finding graphically the intersection with the ordinate which gives the velocity at the time zero. Usually, a function of  $x/t$ , for example  $\log x/t$ , is plotted to obtain straight lines, more suitable for graphical extrapolation than curves with irregularly changing slopes. An example of this procedure is given in Fig. 3. Attention is called to the fact that the decrease of  $\log x/t$  is not regularly a linear function of  $t$ . The graphical extrapolation to the time zero is consequently influenced by a personal factor.

Attempts were made to find other methods for the graphic representation of the experimental data which would result in straight lines and would, therefore, permit a more objective determination of intersections and slopes.

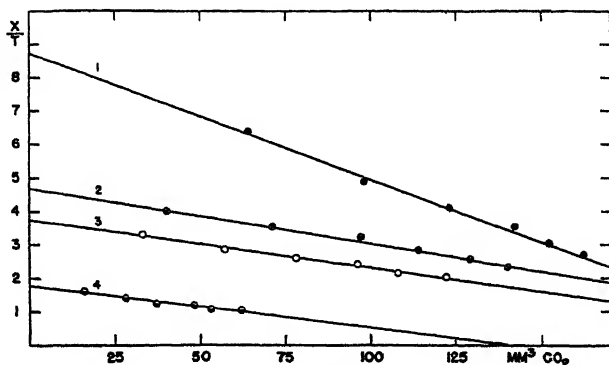


FIG. 4

Graphical Determination of Initial Velocities by Plotting Observed Reaction Velocities against Amounts of  $\text{CO}_2$  formed  
(Same experimental data as used for Figs. 2 and 3)

Since the decrease in reaction velocity seemed to depend on the amount of substrate decarboxylated at any given time, a correlation between  $x/t$  and  $x$  was, therefore, sought. It was found that a straight line resulted when  $x/t$  was plotted against  $x$ . In Fig. 4 this relationship is illustrated, the same experiments which were evaluated for Fig. 3 being used.

The equation for the straight lines in Fig. 4 can be written as

$$\frac{x}{t} = mx + b$$



where  $m$  and  $b$  are constants, representing the slope of the line and the intersection with the ordinate respectively. The reaction velocity at the time zero is found by extrapolation of  $x$  to zero, when no decarboxylation has yet taken place, and can be read off as the point of intersection of the straight line with the ordinate.

The last equation can be rearranged to read:

$$\frac{1}{t} = b \cdot \frac{1}{x} + m$$

This, again, is the equation for a straight line. The slope of the line obtained on plotting  $1/t$  against  $1/x$  is, then,  $b$  or the initial velocity. Fig. 5 shows the lines obtained on plotting  $100/t$  against  $100/x$  with the same experimental data as used for Figs. 3 and 4.

In Table III are listed the initial velocities determined by the three graphical extrapolation methods applied to the data in Fig. 2. The curves are numbered 1-4 in the order of decreasing activity.

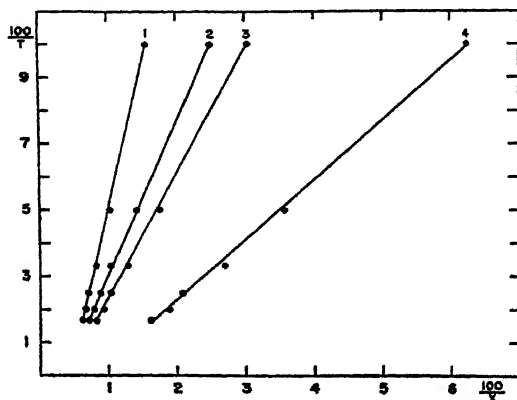


FIG. 5

Graphical Determination of Initial Velocities by Plotting the Reciprocals of Time against the Reciprocals of the Amounts of  $\text{CO}_2$  formed  
(Same experimental data as used for Figs. 2-4)

TABLE III  
*Comparison of Initial Velocities Determined graphically by Three Extrapolation Methods*

Curve No.	Initial velocities in mm <sup>3</sup> . $\text{CO}_2$ obtained by plotting		
	$\log x/t$ vs. $t$	$x/t$ vs. $x$	$1/t$ vs. $1/x$
1	8.91	8.72	8.88
2	4.47	4.66	4.65
3	3.37	3.74	3.76
4	1.95	1.78	1.80

#### 4. Determination of the Michaelis-Menten Constant

Abbreviations:  $(E_T)$  = total enzyme concentration,  
 $(S)$  = substrate concentration,  
 $(ES)$  = concentration of enzyme-substrate complex,  
 $(E_T) - (ES)$  = concentration of free enzyme,  
 $k_s$  = dissociation constant of complex  $ES$ ,  
 $V_m$  = maximal reaction velocity,  
 $v$  = observed (initial) velocity.

Michaelis and Menten (5) formulated a dissociation constant for the enzyme-substrate complex as:

$$k_s = \frac{(S) \times [(E_T) - (ES)]}{(ES)}$$

It is assumed that the rate of the enzymatic reaction is directly proportional to the concentration of  $ES$ . Consequently, the reaction velocity should be  $\frac{1}{2}$  of  $V_m$  if  $\frac{1}{2}$  of the total enzyme is present as  $ES$ , i.e., if  $(ES) = \frac{1}{2}(E_T)$ . The substitution of  $2(ES)$  for  $(E_T)$  changes the equation above to

$$k_s = (S).$$

The numerical value of the Michaelis-Menten constant  $k_s$  is, therefore, the substrate concentration at half-maximum velocity.

Lineweaver and Burk (6) described simple graphical methods for the determination of  $k_s$  from experimental data. Plotting the reciprocals of observed initial velocities against the reciprocals of the corresponding substrate concentrations leads to a straight line with the ordinate intercept  $1/V_m$ , the slope  $k_s/V_m$  and the abscissa intercept  $-1/k_s$ .

A series of experiments with various substrate concentrations was performed and the initial reaction velocities were determined by graphical extrapolation as described in the previous section. The reciprocals of the initial velocities were then plotted against the corresponding  $1/(S)$  values and  $k_s$  was obtained by determining the intersection of the best fitting line with the abscissa. From three independent sets of experiments with carrot extracts,  $k_s$  at pH 5.60–5.70 was found to average  $3.6 \pm 0.4 \times 10^{-3}$  M/l. This is only an approximate value, but the nature of the graphical extrapolation processes did not allow a more precise statement. Taylor and Gale (7) found for glutamic acid decarboxylase from coliform organisms a  $k_s$  of  $5 \times 10^{-3}$  M/l. when the intact organisms were used and  $27 \times 10^{-3}$  M/l. with cell-free ex-

tracts, at pH 4.25. The  $k_s$  values of other cell-free bacterial decarboxylases were (in  $10^{-3}$  M/l.) for lysine: 1.2–1.8, dihydroxyphenyl alanine: 2.3, histidine: 7.5, ornithine: 4 and arginine: 0.75 (7, 8, 9, 10).

### 5. Inhibition of Glutamic Acid Decarboxylase by Hydroxylamine

Okunuki (1) has shown that glutamic acid decarboxylase from plants is inhibited by HCN and a number of aliphatic acids. The degree of inhibition was 89% at a cyanide concentration of  $10^{-4}$  M/l. and 100% at  $10^{-3}$  M/l. As the first step of the enzymatic decarboxylation seems to be a combination between the aldehyde group of pyridoxal phosphate and the amino group of the substrate, it was to be expected that aldehyde reagents such as hydroxylamine would also be inhibitors for glutamic acid decarboxylase.

Clear carrot extracts were prepared as described in the previous report and neutral hydroxylamine solutions of various concentrations were added. The assay for enzymatic activity was carried out as previously described. In all experiments the pH was 5.70, the substrate concentration  $6.8 \times 10^{-3}$  M/l. From the time-activity curves in Fig. 2

TABLE IV  
*Inhibition of Glutamic Acid Decarboxylase from Carrots by Hydroxylamine*

Concentration of hydroxylamine $10^{-3}$ M/l.	Per cent inhibition of initial velocity	
	Extract I-96 $v_0 = 8.9 \text{ mm.}^3 \text{ CO}_2/\text{min.}$	Extract II-2 $v_0 = 14.0 \text{ mm.}^3 \text{ CO}_2/\text{min.}$
3	48–50	46
6	58–62	67
10	78–80	94

it can be seen that the degree of inhibition caused by a given hydroxylamine concentration decreases as the experiment progresses. The effect on the initial velocities of two different enzyme preparations is shown in Table IV. Fig. 6 shows in curve 0 the influence of hydroxylamine on initial velocities and in curve 60 the effect of the inhibitor on the average velocity over a period of 60 min. During the gradual change from curve 0 with a decreasing slope to curve 60 with an increasing slope, there should be a period during which a straight line correlates activity and inhibitor concentration. This is practically the

case at 30 min., as shown in curve 30. The hydroxylamine concentration required for a 50% reduction of the initial velocity was found to be about  $3 \times 10^{-5}$  M/l. A 50% decrease in total  $\text{CO}_2$  production over a period of 60 min. was observed in the presence of  $7-9 \times 10^{-5}$  M  $\text{NH}_2\text{OH}$ /l. Glutamic acid decarboxylase of bacterial origin was inhibited 42% by  $10^{-4}$  M  $\text{NH}_2\text{OH}$ /l. (7), calculated from the amounts of  $\text{CO}_2$  formed during 5 min.

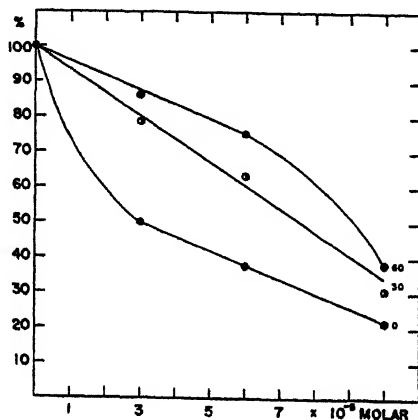


FIG. 6

#### Influence of Hydroxylamine on Reaction Velocities

Curve O: effect on initial velocity; curve 30: effect on average velocity for 30 min. period; curve 60: effect on average velocity for 60 min. period.

### SUMMARY

1. Glutamic acid decarboxylase from carrots showed highest activity between pH 5.30 and 5.90; no activity was observed beyond pH 4.0 and 7.5.

2. The time-activity curve for glutamic acid decarboxylase does not follow the course of a monomolecular reaction curve. A first order curve was, however, obtained on addition of pyridoxal phosphate. The mechanism of this effect of pyridoxal phosphate is at present unknown.

3. The determination of initial velocities by extrapolating  $\log x/t$  plotted against  $t$  to the time zero was found to be subject to personal bias because of the lack of a strict linear relation between the two variables. It was found that straight lines are obtained if velocities

( $x/t$ ) are plotted against the amount of substrate decarboxylated ( $x$ ). The initial velocity in such a graph is given as the intersection of the line with the ordinate. Straight lines were also obtained on plotting  $1/t$  against  $1/x$ . The initial velocities in such graphs are equal to the slopes of the lines.

4. The substrate concentration at which  $\frac{1}{2}$  maximal speed of glutamic acid decarboxylase from carrots is reached, was found to be approximately  $3.6 \pm 0.4 \times 10^{-3}$  M/l. at pH 5.60–5.70.

5. The concentration of hydroxylamine necessary for a 50% decrease in the initial velocity of glutamic acid decarboxylase at pH 5.70 was determined as  $3 \times 10^{-5}$  molar. A concentration of about  $8 \times 10^{-5}$  M hydroxylamine/l. was required to lower the total  $\text{CO}_2$  output over a period of 60 min. to 50% of that produced in the absence of the inhibitor.

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# The Comparative Effect of the Fatty Acids of Butterfat and Corn Oil on the Growth and Metabolism of Microorganisms

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## INTRODUCTION

Weanling rats fed *ad libitum* rations containing lactose and corn oil were found by the Wisconsin group (1, 2) to grow at a rate inferior to that obtained with butterfat. Boutwell *et al.* (3), suggested as an explanation of this observation that the substitution of corn oil for butterfat results in a decreased synthesis of B vitamins by the intestinal flora. More recently (4) the Wisconsin group has indicated that the apparent requirement of the rat for vitamins of the B complex may be altered by a change in the kind of dietary fat even when carbohydrates other than lactose are used in the ration.

To illustrate the probable mode of action of the fats, an *in vitro* experiment which showed the effect of the fatty acids in corn oil and butterfat on the growth of *Streptococcus lactis* and *Lactobacillus casei* was described (4). No implied significance of either organism *in vivo* was intended. However, these investigators pointed out that, if a portion of the vitamin synthesizers or utilizers in the tract were similarly inhibited or allowed to grow, significant differences in the kind and amount of vitamins available to the host would be possible.

Bauernfeind *et al.* (5) found that oleic, stearic and palmitic acids stimulated the production of lactic acid by *L. casei* in the presence of suboptimal amounts of riboflavin or pantothenic acid, while linoleic acid either stimulated or inhibited, depending on the amount used. In the experiments of Strong and Carpenter (6) oleic and stearic acids markedly stimulated the bacterial response in the riboflavin assay with *L. casei*, while palmitic as well as linoleic acid acted as a potent inhibitor. Both stimulation and inhibition of the bacterial response, depending on the level both of vitamin and of fatty acid used, was observed by Neal and Strong (7) with oleic acid in the assay for pantothenic acid. Kodicek and Worden (8), using the same organism, obtained an increase in acid production with the saturated fatty acids stearic and palmitic, and an inhibition of acid production with the unsaturated fatty acids oleic, linoleic and linolenic.

Using *L. arabinosus* as the test organism and assaying for nicotinic acid, Krehl, Strong and Elvehjem (9) found little interference from fatty acids. However, when *L. arabinosus* was used to test for pantothenic acid, an increased acid production was

observed (10, 11, 12). In the microbiological assay for biotin, fatty acids stimulated the bacterial response both when *L. casei* and *L. arabinosus* were used as the test organism (13, 14)

The response of these two microorganisms to different fatty acids is seen, from the above observations, to be quite variable. The following *in vitro* studies were, therefore, conducted to compare the effect of the fatty acids of butterfat and corn oil on the growth or metabolism of seven different microorganisms, with different vitamins and amino acids as the factors limiting growth as well as with a complete medium.

### EXPERIMENTAL

Butterfat and the free fatty acids of butterfat and corn oil were prepared according to standard procedures with special measures taken to prevent oxidation. One gram of butterfat, corn oil or the free fatty acids of each, was dissolved in 100 ml. of aldehyde-free ethanol (95%). Dilutions were then made with distilled water from each of these four stock solutions to obtain final concentrations of 10, 50, 100 and 1000  $\gamma$ /ml.

The microbiological tests were carried out as described in published assay procedures. Solutions of crystalline vitamins and amino acids were used as standards. All assays were run in duplicate.

*L. casei* was used as the test organism to assay for riboflavin by the procedure of Snell and Strong (15), for "folic acid" by the method of Teply and Elvehjem (16) and for pantothenic acid according to the procedure of Neal and Strong (7).

The neutral butterfat and corn oil, at all four concentrations, were tested in the riboflavin and pantothenic acid assays. These exerted no effect on the production of lactic acid in the assay for pantothenic acid, and only a slight effect at the highest level of 1000  $\gamma$ /10 ml. tube was observed in the riboflavin assay, where both fats stimulated the production of lactic acid.

The results of the riboflavin assay are seen in Fig. 1. The most interesting feature of these results is illustrated at the level of 100  $\gamma$  of the free fatty acids. The fatty acids of butterfat greatly stimulated the acid production at the basal level and at 0.05  $\gamma$  of riboflavin and completely inhibited acid production at the three higher levels of the vitamin, whereas corn oil showed the reverse effect and completely inhibited acid production at the lower levels and showed no effect at the higher levels. The fatty acids of corn oil either inhibited the production of lactic acid by *L. casei* in the riboflavin assay or had no effect at all. The fatty acids of butterfat, as well as those of corn oil, com-

pletely inhibited the metabolism of *L. casei* in the riboflavin assay at the level of 1000  $\gamma$  per tube.

In the "folic acid" assay (Fig. 1) crystalline vitamin Bc<sup>1</sup> was used as a standard. Ten  $\gamma$  of butterfat acids had no effect at the basal level but stimulated metabolism at all levels of the vitamin. The fatty acids

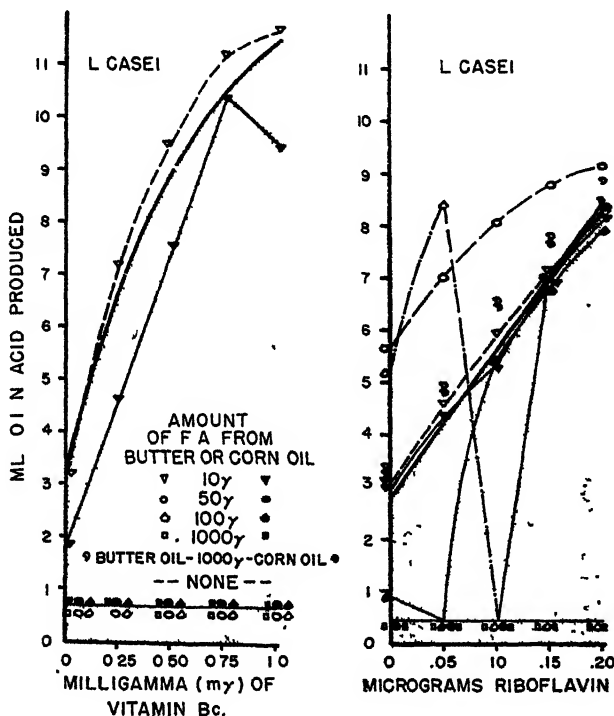


FIG. 1

Acid Production of *L. casei* in Response to Graded Amounts of Vitamin Bc and Riboflavin in the Presence of the Free Fatty Acids of Butterfat and Corn Oil.

in corn oil inhibited acid production at the 10  $\gamma$  concentration. All higher levels of both fats completely inhibited the acid production by *L. casei*.

Pantothenic acid was assayed using both *L. casei* and *L. arabinosus* (Fig. 2). In the *L. casei* assay the fatty acids of butterfat had no effect

<sup>1</sup> We are indebted to Parke, Davis and Company, Detroit, Michigan, for the crystalline vitamin Bc used in these studies.



at the 10  $\gamma$  level, whereas the fatty acids in corn oil greatly stimulated metabolism at this concentration. As in the assay for riboflavin, the highest level of both fats also inhibited the metabolism of *L. casei* in the assay for pantothenic acid, but at the lower levels the fatty acids from corn oil stimulated acid production.

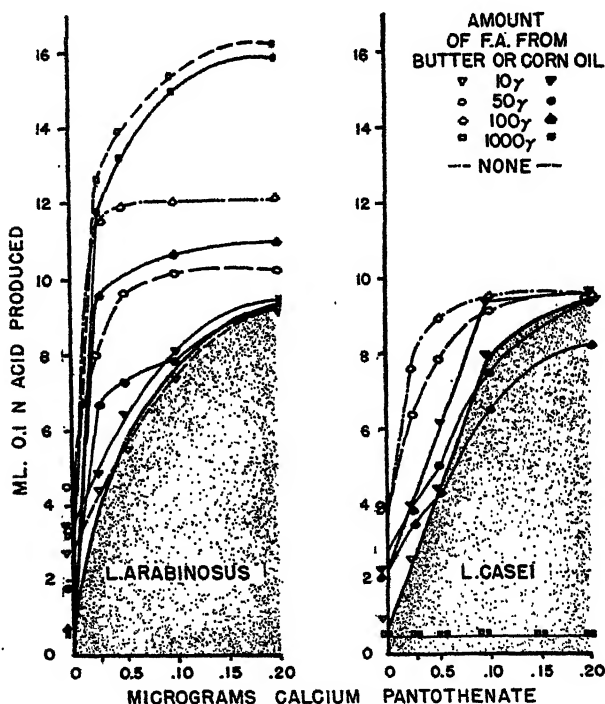


FIG. 2

Acid Production of *L. casei* and *L. arabinosus* in Response to Graded Amounts of Pantothenic Acid in the Presence of the Free Fatty Acids of Butterfat and Corn Oil.

Asaying for pantothenic acid with *L. arabinosus*, a 10  $\gamma$  concentration of the fatty acids of corn oil stimulated acid production at all levels of the vitamin. The same concentration of the fatty acids in butterfat showed no effect except for a slight stimulation at the basal level. The highest level of 1000  $\gamma$  of the free acids from both fats gave great stimulation, contrary to the complete inhibition observed with *L. casei*. No inhibition at all was observed with *L. arabinosus*.

The nicotinic acid assay with *L. arabinosus* was completely unaffected by either fatty acids (Fig. 3).

Three mutant strains of *Escherichia coli* were obtained through the generosity of Dr. J. O. Lampen. The nicotinamide assay was carried out as reported by Roepke *et al.* (17) and the amino acid assays according to the procedures of Lampen *et al.* (18, 19).

The concentration of alcohol used to dissolve the fatty acids was tested with all organisms. *L. casei* and *L. arabinosus* were unaffected by the concentrations of alcohol used. All three mutant strains of *E. coli* showed a growth inhibition with the highest concentration of alcohol (0.95%) (Figs. 4, 5). The growth of the mutants requiring

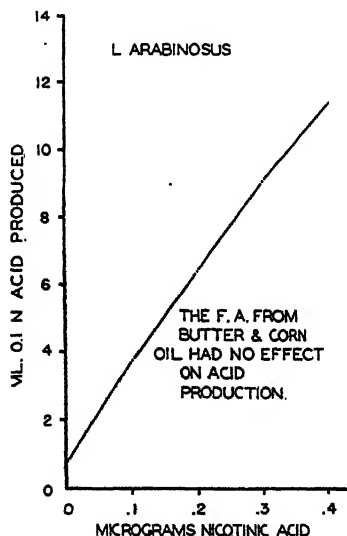


FIG. 3

Acid Production of *L. arabinosus* in Response to Graded Amounts of Nicotinic Acid in the Presence of the Free Fatty Acids of Butterfat and Corn Oil.

tryptophan and arginine was only slightly inhibited, whereas that of the mutant requiring nicotinamide was inhibited to a much larger extent by the alcohol.

The mutant requiring nicotinamide was the most sensitive to the effect of the fatty acids from butter and corn oil. At the 10  $\gamma$  level neither the fatty acids of butterfat nor of corn oil had any effect on

growth at the three lower levels of the vitamin and both inhibited to an equal extent at the two higher levels. With 50  $\gamma$  and 100  $\gamma$  the fatty acids of butterfat inhibited at all levels of the vitamin except the basal with no added nicotinamide, whereas the fatty acids of corn oil inhibited only at the two higher levels of the vitamin with 50  $\gamma$  and the three higher levels with 100  $\gamma$ . At the latter concentration of the fatty acids of corn oil there was a slight stimulation of growth when no nicotinamide was added to the basal medium. This stimulation was even greater when 100  $\gamma$  of the fatty acids of corn oil were added and extended to the level of .025  $\gamma$  of nicotinamide. No effect was observed

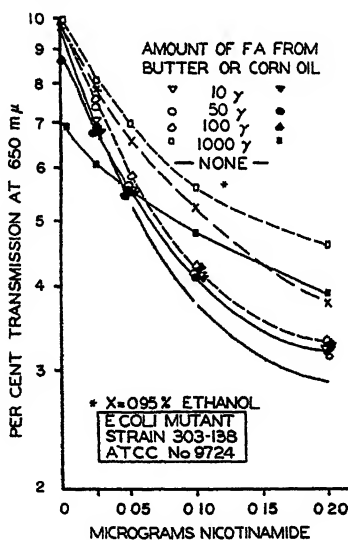


FIG. 4

Growth of *E. coli* mutant in Response to Graded Amounts of Nicotinamide in the Presence of the Free Fatty Acids of Butterfat and Corn Oil. Per cent transmission, as a measure of growth, is given in units of 10.

at the intermediate level and an inhibition which was less, or not significantly greater, than the inhibitory effect of 0.95% alcohol alone, was observed at the two higher levels of the vitamin. On the other hand, 1000  $\gamma$  of the fatty acids of butterfat inhibited the growth of this mutant to a greater extent than ethanol alone, at all levels of the vitamin.

The mutants requiring either arginine or tryptophan were un-

affected by 10 or 50  $\gamma$  of the fatty acids of either butterfat or corn oil. The arginine-requiring mutant was inhibited only slightly by 100  $\gamma$  and to a greater extent by 1000  $\gamma$  with the fatty acids of butterfat producing a slightly lesser inhibition than the fatty acids of corn oil. The mutant requiring tryptophan was inhibited to a greater extent

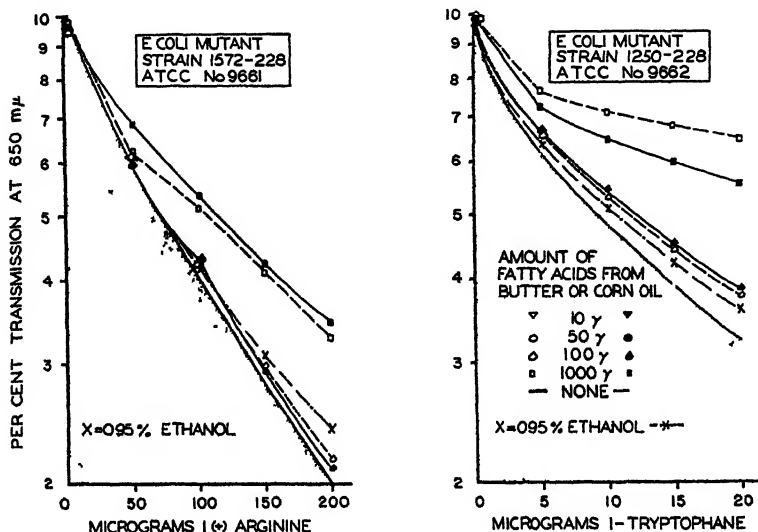


FIG. 5

Growth of *E. coli* mutant in Response to Graded Amounts of *L*(+)-Arginine and *L*-Tryptophane in the Presence of the Free Fatty Acids of Butterfat and Corn Oil. Per cent transmission, as a measure of growth, is given in units of 10.

by both these levels of fatty acids and at the highest level of 1000  $\gamma$  the fatty acids of butter fat inhibited growth to an appreciably greater degree than the fatty acids of corn oil.

Two "parent" cultures of *E. coli*<sup>2</sup> and the three mutant strains of *E. coli* were grown on a complete medium. The medium consisted of Difco A. C. Broth Experimental which was extracted three times with ethyl ether and autoclaved for 20 minutes to drive off the ether. One ml. of supplement (test material) was added to 9 ml. of medium. The tubes were incubated for 7 hours and 20 minutes at 37°C. The tubes were then autoclaved for 15 minutes to stop further growth, and cooled before reading the turbidity in a Coleman Universal Spectrophotometer, Model 11, at 650  $m\mu$ . The uninoculated blanks were set at 100. The results are given in Table I.

<sup>2</sup> These cultures of *E. coli* were obtained through the courtesy of Dr. F. P. Clark of the Bacteriology Department. Culture 251 is A. T. C. C. No. 4348 and Culture 252 was originally obtained from C. E. A. Winslow of Yale.

TABLE I

*The Effect of Ethanol and of the Fatty Acids of Butterfat and Corn Oil on the Growth of Five Strains of E. Coli on a Complete Medium<sup>1</sup>*

Galvanometer reading as a measure of growth

Supplement	<i>E. coli</i> 251	<i>E. coli</i> 252	<i>E. Coli</i> mutant requiring		
			Nicotinamide	Arginine	Tryptophan
None (control)	21	14	12	12	11
0.95% ethanol	33	19	12	12	12
	B. C.O.	B. C.O.	B. C.O.	B. C.O.	B. C.O.
Neutral fat 1 mg.	40 54	19 24	16 19	16 20	15 17
Free fatty acids 10 $\gamma$	24 29	14 14	12 12	12 12	11 12
Free fatty acids 50 $\gamma$	24 28	14 13	12 12	12 12	11 11
Free fatty acids 100 $\gamma$	31 30	15 14	14 12	13 12	13 11
Free fatty acids 1000 $\gamma$	56 55	23 19	18 19	21 15	16 15

<sup>1</sup> Ether extracted—Difco A. C. Broth Experimental

B = Butterfat. C.O. = Corn Oil.

Alcohol, strangely enough, had no effect on the three mutant strains grown on the complete medium, contrary to the inhibition observed with the synthetic media. A very slight growth depression of questionable significance was observed with culture 252, and a greater effect was found with culture 251.

In the tests with 1 mg. of the neutral fat, again the greatest effect produced was on culture 251 with a lesser effect on the mutant strains. In all cases corn oil depressed growth to a greater extent than butter oil.

The three lower levels of the free fatty acids of both fats likewise had no effect on the mutant strains or culture 252. All levels of the free fatty acids depressed the growth of culture 251. At the lower levels of 10 and 50  $\gamma$  the free fatty acids of corn oil exerted a greater growth inhibitory action on culture 251 than did those of butter while at the levels of 100 and 1000  $\gamma$  there were no differences in the extent of inhibition produced. At the 1000  $\gamma$  level of the fatty acids those of butterfat exerted a greater inhibition than those of corn oil in *E. coli* 252 and in the mutant requiring arginine. In both the mutant requiring nicotinamide and tryptophan the inhibition was of equal extent.

In a preliminary experiment with a yeast requiring thiamine, 1 mg. of the fatty acids both from butter and corn oil completely inhibited growth of this organism in the presence of suboptimal amounts of thiamine.

## DISCUSSION

The effect of the fatty acids of butterfat and corn oil have been compared in a few representative assays. The results indicate clearly that the effect produced by the fatty acids is dependent not only upon the microorganism tested but also upon the level of the vitamin or amino acid limiting growth as well as the level of the fatty acids themselves. It is unjustifiable, therefore, to generalize that the fatty acids of any fat or oil are superior nutritionally to those found in another fat or oil from any stimulation or inhibition of microbiological growth or metabolism obtained with a few organisms under special conditions.

If it were presumed that these results may be indicative of the action of fatty acids on microorganisms *in vivo*, the data obtained with *E. coli* would have the greatest significance because *E. coli* is normally a dominant organism in the human intestinal flora. The results of the tests with the mutant strains of *E. coli* grown on the basal medium show that the fatty acids of corn oil do not limit the growth of these organisms to the same extent as the fatty acids of butterfat. The tests with these mutant strains as well as with two "parent" strains of *E. coli* grown on a complete medium, however, revealed that in only 2 of the 5 organisms did the free fatty acids of butterfat exert a greater growth inhibitory action than did those of corn oil and this only at the highest level of 1000  $\gamma$ . The neutral fats produced the reverse picture of a greater inhibition by corn oil.

These results were obtained for the most part on organisms which have a specific requirement for a certain vitamin or amino acid which was made the growth-limiting factor. Thus any effect exerted by the fatty acids in these tests might have a counterpart *in vivo* on the vitamin utilizers. No tests have yet been reported which indicate that a similar effect is exerted on microorganisms which are vitamin synthesizers.

Williams (13), in seeking an explanation for the high acid and cell production in the presence of basal medium and rice oil without added pure biotin, tested for the possibility of biotin synthesis, but was unable to demonstrate any evidence of such synthesis.

Kodicek and Worden (8) point to the fact that reversal can be brought about by lecithin or cholesterol after inhibition for 48 hours by linoleic acid as indicating that the action is bacteriostatic. They have suggested that the phenomenon may be a direct chemical action

upon the metabolism of the bacteria, or upon the availability of some metabolite present in the medium, although they favor a physico-chemical action as an explanation.

The relationship of turbidity to acid production by *L. arabinosus* is of interest in considering the difference in response of this organism to fatty acids depending upon whether pantothenic acid or nicotinic acid is the limiting growth factor. The turbidity produced by the growth of *L. arabinosus* was found by Isbell (20) to be greater in relation to acid production when the organisms are responding to suboptimal amounts of nicotinic acid than when they are responding to suboptimal amounts of biotin, pantothenic acid or *l*-tryptophan.

The data presented here serve to emphasize the inadvisability (6) of adding fatty acids to the basal medium to overcome the production of drifts in the assay of fatty materials.

I wish to thank Dr. H. H. Mitchell for the suggestion which was responsible for the undertaking of this investigation.

#### SUMMARY

The effect of the fatty acids of butterfat and corn oil on the growth or metabolism of seven different microorganisms was compared, at the levels of 10, 50, 100 and 1000  $\gamma$ , with different vitamins and amino acids as the factors limiting growth as well as with a complete medium. The effect produced by the fatty acids is dependent not only upon the test microorganism but also upon the level of the vitamin or amino acid limiting growth as well as the level of the fatty acids themselves. In the tests described there was no consistent distinction between the fatty acids of butterfat and of corn oil in their effects upon bacterial growth, and presumably upon bacterial synthesis of vitamins.

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# Thermal Destruction of Influenza A Virus Hemagglutinin. III. The Effect of Urea<sup>1</sup>

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## INTRODUCTION

Since the report of Hopkins (1) that egg albumin is denatured in concentrated urea solutions, denaturation by this agent has been shown to be a characteristic of several other proteins and several viruses (2, 3, 4). The urea denaturation of proteins is of peculiar interest because of its temperature coefficient. Hopkins (1) found a negative coefficient for the derivative of the velocity of urea denaturation of egg albumin with respect to temperature. Stanley and Lauffer (3) showed that the differential temperature coefficient of the urea disintegration of tobacco mosaic virus could be either positive or negative, depending upon the temperature range under consideration. Bawden and Pirie (4) found the same phenomenon exhibited in the urea denaturation of several other viruses. More recently, Clark (5) found, contrary to Hopkins' observation, a positive differential temperature coefficient for the denaturation of egg albumin. Therefore, in the course of investigations on the thermal stability of the hemagglutinin of PR-8 influenza virus (6, 7) it appeared of interest to determine the effect of urea.

## EXPERIMENTAL

*Materials and Methods:* The preparation of the sample of virus, the method of determining chicken red cell agglutinating activity (CCA), and the calculation of the velocity constant for the reaction, were previously described (6). In the present experiments, urea was added in required amounts immediately before the virus sample was placed in the heated water bath. All virus samples had been previously dialyzed against a phosphate buffer of pH 7.1 and ionic strength 0.2.

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<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pittsburgh.

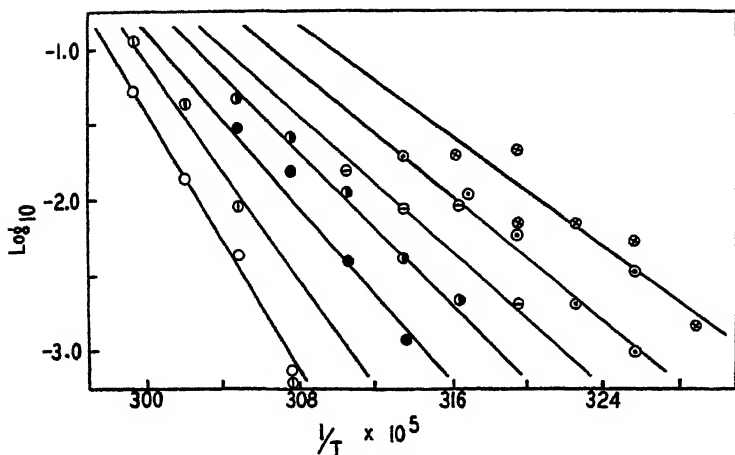


FIG. 1

Log<sub>10</sub> of  $k$  Plotted against Reciprocal of Absolute Temperature for Various Urea Concentrations

⊕—corresponds to 4.08  $M$  urea, ○—3.51  $M$ , ⊖—2.90  $M$ , ●—2.25  $M$ , ●—1.55  $M$ , ⊙—.80  $M$ , ○—no urea.

**Results:** The log reaction velocity constant of the destruction of CCA activity is plotted in Fig. 1 as a function of the reciprocal of absolute temperature for the urea concentrations used. On the assumption that the Arrhenius equation holds, lines have been drawn to approximate these data. From the slope of these lines, the energy of activation ( $E$ ) was calculated. The heat of activation,  $E-RT$ , symbolized by  $\Delta^*H$ , is plotted in Fig. 2 as a function of log urea concentration.

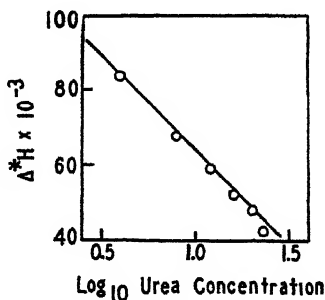


FIG. 2

Heat of Activation Plotted as a Function of log of Urea Concentration

If the Eyring equation,

$$RT \ln k \frac{h}{k'T} = -\Delta^*H + T\Delta^*S \quad (1)$$

(in which  $k$  is the reaction velocity constant,  $\Delta^*H$  is the heat of activation equal to  $E-RT$ ,  $\Delta^*S$  is the entropy of activation,  $T$  is the absolute temperature,  $R$  is the gas constant,  $h$  is Planck's constant and  $k'$  is the Boltzmann constant) is assumed to apply, it is found, as shown in Fig. 3, that the calculated value of  $\Delta^*S$  is also a linear function of log molar urea concentration (Fig. 3). Accordingly, equation (1) can be

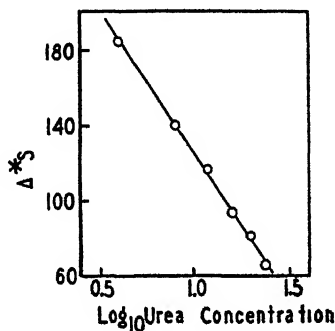


FIG. 3

Entropy of Activation Plotted as a Function of log of Urea Concentration

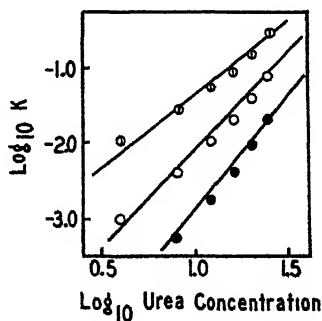


FIG. 4

$\log_{10}$  of Reaction Velocity Constant Plotted as a Function  
of  $\log_{10}$  Urea Concentration

○—55°C., ○—44°C., ●—43°C.

modified to express the effect of urea concentration  $[U]$  and temperature on the reaction velocity constant,

$$RT \ln k \frac{h}{k'T} = -\Delta^*H' + b \log [U] - T(\Delta^*S' - c(\log [U])). \quad (2)$$

The values of  $\Delta^*H'$  and  $b$  are the intercept and slope in Fig. 2;  $\Delta^*S'$  and  $c$  the corresponding values in Fig. 3.

In this equation, it is seen that the log reaction velocity constant should be a linear function of the log urea concentration at any given temperature. This appears to be confirmed in Fig. 4, which shows the relation of log reaction velocity constant to log urea concentration at different temperatures. The points are taken from Fig. 1, and the lines are calculated from equation (2).

### DISCUSSION

It can be seen from Fig. 3 that the entropy of activation decreases with increasing urea concentration. Entropy can be interpreted as a measure of the probability of the particular state of a substance as derived from statistical mechanics. A high entropy corresponds to a highly probable state—that is, one of high randomness or low degree of organization. The entropy of activation is the theoretical change in entropy experienced when a substance is transformed from the normal stable state to the activated or reactive state. The fact that the activation process in this study has a positive entropy change can be considered as meaning that the activated state of the hemagglutinin is more probable or more random or less organized than the normal state. That is, the hemagglutinin has more degrees of freedom or fewer restrictions in the activated than in the normal state. This increase in degrees of freedom could be due to the breaking of internal bonds or linkages in the activation process. When urea is added, the entropy change in the activation process is decreased. One interpretation of this decrease in entropy change is that, in the presence of urea, the normal state of the virus has a higher entropy than in the absence of urea. This would account for the lower increase in passing to the activated state. From this, one might infer that the action of urea on the hemagglutinin is to break internal linkages in a reversible manner. This concept of the action of urea is consistent with the idea advanced on previous occasions that urea forms reversible complexes with proteins and viruses.

If disintegration or denaturation in the presence of urea is due to an irreversible change in a reversible virus-urea complex, it follows from almost any modern theory of reaction kinetics and from the law of mass action that the log rate of the overall process will be a linear function of the log urea concentration. Thus, the linear relationship shown in Fig. 4 can be interpreted as indicating that the rate of inactivation of virus is related to the concentration of virus in the urea complex form. Even though this result might be considered as evidence for the formation of urea virus complexes, it contributes no information on the role of urea in decreasing the stability of the virus.

The idea that urea forms reversible complexes with proteins and viruses was first advanced by Hopkins to account for the negative differential temperature coefficient of the rate of urea denaturation of egg albumin. Hopkins postulated that, in the denaturation process, albumin reacted with urea to form a complex which then denatured, and that the influence of temperature upon the dissociation of the complex is greater than that upon the velocity of the irreversible change of the complex to denatured albumin. This idea was extended by Lauffer (8), who postulated the existence of at least two and possibly many different urea-protein complexes in the case of tobacco mosaic virus. At least one of these complexes was assumed to be of such a nature that the effect of temperature on reversible dissociation is greater than upon rate of irreversible destruction of the complex, and at least one complex was assumed to be of such a nature that the effect of temperature upon the irreversible destruction was greater. This extension of Hopkins' idea was devised to explain the change from negative to positive differential temperature coefficient of tobacco mosaic virus destruction as temperature is elevated.

### SUMMARY

The thermal stability of the hemagglutinin of PR-8 influenza virus in the presence of urea has been studied. The energy and calculated entropy of activation of the thermal inactivation process are inverse linear functions of log urea concentration.

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# Thermal Destruction of Influenza A Virus Hemagglutinin. IV. The Effect of Initial Virus Concentration<sup>1</sup>

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## INTRODUCTION

Miller (1) observed that the stability of influenza virus hemagglutinin at 4° and 23°C. seemed to be greater in initially more concentrated solutions than in initially more dilute solutions. Lauffer and Carnelly (2) found that at 61°C. influenza A virus hemagglutinin was destroyed more rapidly in initially dilute solutions than in initially concentrated solutions.

The thermal destruction of hemagglutinin was found by Lauffer and Carnelly (2) to follow the course of a three halves order reaction. It was suggested that this behavior might be an artifact resulting from an inhomogeneity of the virus with respect to ease of destruction of hemagglutinin. If this is really the case, it can be shown on simple theoretical grounds that the reaction velocity constant calculated by the law of a three halves order reaction should vary with initial concentration. It was thought worthwhile, therefore, to investigate more fully the dependence of the rate of destruction of hemagglutinin upon initial virus concentration.

## MATERIALS AND METHODS

A fairly concentrated virus solution (4000 CCA units/cc.) was dialyzed against a phosphate buffer of pH 7.1 and ionic strength 0.2. The virus was diluted as required with this buffer. The method of determining chicken red cell agglutinating (CCA) activity and the calculation of the velocity constant of the inactivation reaction were previously described (2).

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<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pittsburgh.



## RESULTS

The results are presented in Table I. There is a fairly regular increase of the reaction constant with decrease in concentration, in general agreement with the results of Lauffer and Carnelly (2). As can

TABLE I  
*Reaction Velocity Constants of Thermal Inactivation of CCA as a  
Function of Initial Virus Concentration*

Temp. °C.	55		58		61		64	
$V_0$	$k$	$k\sqrt{V_0}$	$k$	$k\sqrt{V_0}$	$k$	$k\sqrt{V_0}$	$k$	$k\sqrt{V_0}$
1.00	.00155	.00153	.00110	.00110	.0048	.0048	.0353	.0353
0.50	.00038	.00027	.00073	.00051	.0033	.0023	.0427	.030
0.25	.00227	.00113	.00234	.00117	.0065	.0033	.0309	.0155
0.12	.00159	.00056	.00248	.00088	.0216	.0076	.1573	.056
0.06	.00246	.00061	.00432	.00108	.0117	.0029	.334	.083
0.03	.00309	.00055	.00602	.00106	.0187	.0033	.0804	.0142

be seen in Table I, if the reaction constant ( $k$ ) is multiplied by the square root of initial virus concentration, ( $V_0$ ), the dependence of the reaction constant on initial virus concentration is no longer apparent.

If the log of the expression  $k\sqrt{V_0}$  is plotted against the reciprocal of

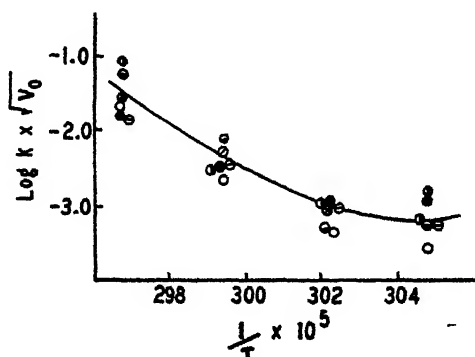


FIG. 1

Log  $k\sqrt{V_0}$  Plotted as a Function of Reciprocal of Absolute Temperature

Data obtained with initial relative concentrations:  $\circ$ —1.00,  $\circ$ —0.50,  $\bullet$ —0.25,  $\circ$ —0.12,  $\bullet$ —0.06,  $\circ$ —0.03.

absolute temperature,  $1/T$ , as in Fig. 1, the points tend to fall about a curve. Thus  $E$ , the Arrhenius activation energy in this experiment seems to vary with temperature. This result seems to be somewhat at variance with those obtained earlier by Lauffer and Scott (3). More data are brought to bear in this instance than previously. If the log of  $k$  is plotted against log of  $V_0$ , as in Fig. 2, straight lines with a slope of  $-.56$  can be shown to approximate the data at various temperatures. This substantiates the dependence of  $k$  upon  $\sqrt{V_0}$  indicated by the data in Table I.

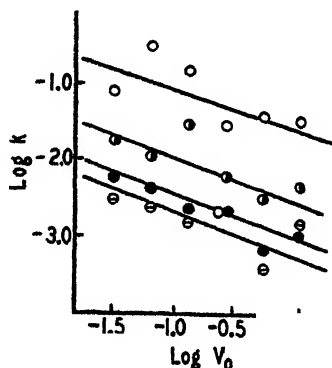


FIG. 2

Log  $k$  Plotted against log of Original Concentration

○—data obtained at 55°C., ●—58°C., ▲—61°C., ○—64°C.

## DISCUSSION

Since most thermal denaturations of pure proteins under well-controlled conditions approximate first order reactions, the three halves order reaction reported for destruction of CCA can be considered as evidence of a multi-component system. The dependence of  $E$  on temperature, which is not always so apparent as in this experiment, can be considered as additional evidence of the complexity of the system.

These kinetic anomalies can be explained if it is assumed that CCA activity is composed of two or more entities with different thermal inactivation characteristics. If it is assumed that two entities are present, and that their inactivation is monomolecular,  $dx/dt = -k_x x$  and  $dy/dt = -k_y y$ , where  $x$  is the amount of one component and  $y$  is the amount

of the other and  $k_x$  and  $k_y$  are first order reaction constants, respectively.

According to Lauffer and Carnelly (2),  $\frac{dV}{dt} = -kV^{\frac{1}{2}}$ . Upon integration

this gives,  $k = \frac{2}{t} \left( \frac{1}{\sqrt{V}} - \frac{1}{\sqrt{V_0}} \right)$ , where  $V_0$  is original virus concentra-

tion. It follows from the assumptions made previously that  $x = x_0 e^{-k_x t}$ ,  $y = y_0 e^{-k_y t}$  and  $x_0 + y_0 = V_0$ , where  $x_0$  and  $y_0$  are original concentrations of the two types of CCA activity. For an initial virus concentration of  $V_0$ :

$$k = \frac{2}{t} \left( \frac{1}{\sqrt{x_0 e^{-k_x t} + y_0 e^{-k_y t}}} - \frac{1}{\sqrt{x_0 + y_0}} \right)$$

and for an initial virus concentration of  $aV_0$ ,

$$k_a = \frac{2}{t} \left( \frac{1}{\sqrt{a} \sqrt{x_0 e^{-k_x t} + y_0 e^{-k_y t}}} - \frac{1}{\sqrt{a} \sqrt{x_0 + y_0}} \right)$$

and thus  $k = \sqrt{a} k_a$ .\*

Thus, if the apparent reaction velocity constant of the thermal destruction of influenza virus is derived from the assumption that a three halves order reaction is involved, while in reality first order reactions of two or more components are taking place, then the apparent constant should vary as the reciprocal of the square root of the initial concentration. The fact that the experimental results are consistent with the theory constitutes evidence that the hemagglutinin activity of influenza A virus does consist of more than one component, as postulated previously (2).

If all the components had the same energy of activation but differed only in an entropy term, one should expect the rates of the individual reactions to vary in the same way with temperature, and the overall process should, therefore, vary with temperature according to the Arrhenius equation. Conversely, if the energies of activation of the components differ significantly, the effect of temperature upon each component velocity will be different. This will tend to cause the temperature response of the overall reaction velocity to depart from the course predicted by the Arrhenius equation. It also could alter the apparent three halves order of the reaction. The results shown in Fig.

\* The same relation would result if more than two components were considered to be present.

1 tend to be consistent with the assumption that the energies of activation of the individual processes are different. However, results obtained earlier do not bring out clearly such a variation (3). The observation reported earlier that the apparent three halves order reaction did not represent the data faithfully at higher temperatures is also consistent with the assumption of a multi-component hemagglutinin with various activation energies.

### SUMMARY

The effect of initial virus concentration on the rate of thermal inactivation of influenza A virus hemagglutinin was studied. It was found that the rate varied inversely as the square root of the initial concentration and that the temperature dependence of the rate did not follow the course prescribed by the Arrhenius equation. These findings and the apparent three halves order of the reaction can be explained in terms of a multi-component system, whose individual components are inactivated according to the first order law.

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# Molecular Weight by Solubility Measurements

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Some 20 years ago the author published a short paper on the ionization of proteins (1). During the time which has elapsed since then, the problems discussed in this paper were reconsidered at intervals by the author (see (2)) and in 1929 a more generalized theory was developed which led to some simple relationships between mean valency and hydrogen ion activity. However, the implications of the formulae derived were not quite clearly seen by the author at that time, other work interfered and it was not until the spring of 1939 that part of this—indeed very elementary—theory was made known to a larger audience (seminar of Harvard Medical School, see (3)). During the war an admirable contribution was made by Jannik Bjerrum ((4), 1941)—in fact, a treatment of the problems in question which had a broader viewpoint than the discussion by the author (which dealt exclusively with the exchange of hydrogen ions between polyvalent ampholytes and solvent). In 1943 Bjerrum (5) followed up his theoretical observations and gave an interesting treatment of the problem of association in general. As regards other contributions (Kirkwood and Wyman (6)) reference is made to the excellent book by Cohn and Edsall (3).

The question which will be the subject of a brief discussion in the present paper was outlined by the author in a paper by Grönwall (7). A protein (or any polyvalent ampholyte) in its pure crystalline state is in equilibrium with an aqueous solution containing, besides the protein, salt, acid or base. If the concentration of either of the two latter components is varied, a solubility curve is obtained from which the molecular weight of the protein may be derived, *viz.*, by means of the expression

$$M_p = - \frac{1}{\bar{h}} \left( \frac{d \log S}{d p a_H} \right)^2 \quad (1)$$

$$p a_H = - \log a_H.$$

In this equation  $S$  is the total quantity of protein dissolved (g./l.),  $M_p$  the molecular weight,  $\bar{h}$  the number of hydrogen ions (g. equivalents) taken up ( $\bar{h}$  positive) or given off ( $\bar{h}$  negative) by 1 g. of protein at the given hydrogen ion activity and protein concentration. The validity of (1) is based on the following conditions:

1. The chemical potential of the protein must be constant, *i.e.*, the composition of the crystals must not change with  $a_H$ , the hydrogen ion activity.

2. The protein must be monodisperse with the same molecular size at all  $pa_H$ -values.

3. The salt concentration must be constant and so large that the activity coefficients of the protein ions formed are constant irrespective of the changes in protein concentration and hydrogen ion activity.

(1) is derived in the following way (see (7)):

In a protein solution at a given  $pa_H$  we must reckon with the presence of a number of different molecules. First of all, there will be several types of molecules with net charge zero, in fact a great number of different amphi-ions which are in mutual equilibrium, one being formed from another by displacement of one or more hydrogen ions within the molecule. The relative concentrations of these different types of molecules depend upon the dissociation constants of the ionogen groups, but are independent of  $pa_H$ . If one is constant, because the molecule in question is in equilibrium with a solid protein phase of fixed structure, all the other concentrations will also be constant. Secondly, there will be several types of molecules with net charge  $+1$ , or with net charge  $+2$ ,  $+3$ , *etc.*, or  $-1$ ,  $-2$ ,  $-3$  *etc.* These groups of molecules are in equilibrium with the (neutral) amphi-ions and the hydrogen ions and, if the activity of the latter is changed by adding acid and base, the activities of the group of amphi-ions will change unless the solution is in contact with solid protein, in which case protein will enter or leave the solution in order to maintain a constant activity. If by  $c_i$  we denote the sum of the concentrations of all ions with net charge  $i$ , and by  $a_0$  the activity of one of the molecules with net charge zero, we have in general

$$c_i = \sum_k c_{ik} = a_0 \cdot a_H^i \sum_k \frac{k_{ik}}{f_{ik}} \quad (2)$$

where  $k_{ik}$  is the constant for the dissociation of the molecules with activity  $a_0$  into one ion with charge  $i$  and of type  $k$ , and  $i$  hydrogen ions, and  $f_{ik}$  is the activity coefficient and  $a_H$  the hydrogen ion activity;  $i$  is reckoned with sign. Inserting for the sake of simplicity

$$\sum_i \frac{k_{ik}}{f_{ik}} = \frac{k_i}{f_i} \quad (3)$$

we obtain

$$c_i = \frac{k_i}{f_i} a_H \cdot a_0 \quad (4)$$

and, in saturated solutions,

$$s = \sum_i c_i = a_0 \sum_i \frac{k_i}{f_i} a_H^i \quad (5)$$

where  $s$  is the solubility and  $a_0$  is constant. By differentiation of (5) we obtain

$$\frac{ds}{da_H} = a_0 \sum_i \frac{k_i}{f_i} a_H^{i-1} i - a_0 \sum_i \frac{k_i}{f_i} a_H^i \frac{d \ln f_i}{da_H} \quad (6)$$

$$\frac{d \ln s}{d \ln a_H} = \frac{a_0 \sum_i \frac{k_i}{f_i} a_H^i i}{a_0 \sum_i \frac{k_i}{f_i} a_H^i} - \frac{a_0 \sum_i \frac{k_i}{f_i} a_H^i \frac{d \ln f_i}{d \ln a_H}}{a_0 \sum_i \frac{k_i}{f_i} a_H^i} = \frac{\sum_i c_i i}{\sum_i c_i} - \frac{\sum_i c_i \frac{d \ln f_i}{d \ln a_H}}{\sum_i c_i}.$$

Hence

$$\left( \frac{d \ln s}{d \ln a_H} \right)_{a_0} = \bar{i} - \bar{\varphi}_i \quad (7)$$

where

$$\bar{i} = \frac{\sum_i c_i i}{\sum_i c_i} \quad (8)$$

is the mean valency, (1), and

$$\bar{\varphi}_i = \frac{\overline{\frac{d \ln f_i}{d \ln a_H}}}{\quad} \quad (9)$$

is the mean dependency upon  $a_H$  of the activity coefficients ( $a_0 = \text{constant}$ ).  $\bar{\varphi}_i$  is an extremely complicated term (especially if the different  $f_{ik}$  have different values) but, provided condition 3 is fulfilled, one is



permitted to discard it, whereby (7) is changed into

$$\left( \frac{d \ln s}{d \ln a_H} \right)_{a_0} = \bar{\tau} \quad (10)$$

It will be observed that in (5)  $s$  is defined on a molar basis,  $c_i$  being the concentration of the particles with the charge  $i$ . In (10) however,  $s$  may be given in any unit whatsoever since multiplying  $s$  by a constant does not change the equation, and since  $\bar{\tau}$  is connected with  $\bar{h}$  by the equation

$$\bar{\tau} = \bar{h} M_p \quad (11)$$

we get

$$\frac{d \ln S}{d \ln a_H} = \bar{h} M_p \quad (12)$$

where  $S$  is expressed as g. of protein/l. and (12) is identical with (1). The question is now with what accuracy the determination of  $S$ ,  $\bar{h}$  and  $pa_H$  can be made.  $S$  should be easy to determine and so also should  $\bar{h}$  if the concentration of free hydrogen ions is small as compared to the quantity of acid added.

In this case we have

$$\bar{h} = c_{\text{acid}}/S \quad (13)$$

where  $c_{\text{acid}}$  is expressed as equivalents/l. The determination of  $pa_H$ , however, meets with the well-known difficulty connected with the formation of diffusion potentials at the liquid junctions of the cells used in the measurements. The accuracy of the  $pa_H$ -determination will, therefore, be the limiting factor in the experimental investigation of the validity of (1).

The experimental material available in the literature permits no accurate verification of (1), but the measurements of the solubility of lactoglobulin by Grönwall may serve as an illustration of its application.

Since the amounts of acid and base added are not given by Grönwall it is convenient to state (1) in another form. Introducing

$$\frac{d\bar{h}}{d pa_H} = -q \quad (q \text{ constant}), \quad (14)$$

verified for lactoglobulin by Cannan, Palmer and Kibrick (8) and Grönwall (9), leads to

$$\frac{d \log S}{d pa_H} = M_p q (pa_H - pa_H^0) \quad (15)$$

( $d \log S / d pa_H = 0$  at the isoelectric point,  $pa_H^0$ ) and

$$\log \frac{S}{S_0} = M_v \cdot \frac{q}{2} (pa_H - pa_H^0)^2 \quad (16)$$

or

$$\log \frac{S}{S_0} = \sqrt{M_v \cdot \frac{q}{2}} (pa_H - pa_H^0), \quad (17)$$

$S_0$  being the solubility at  $pa_H^0$ .

Plotting  $\sqrt{\log S/S_0}$  against  $pa_H$  should then give straight lines, the slopes of which will determine  $\sqrt{M_v q/2}$  and ultimately  $M_v$ , if  $q$  is known. Fig. 1 shows the results of some calculations made on the basis

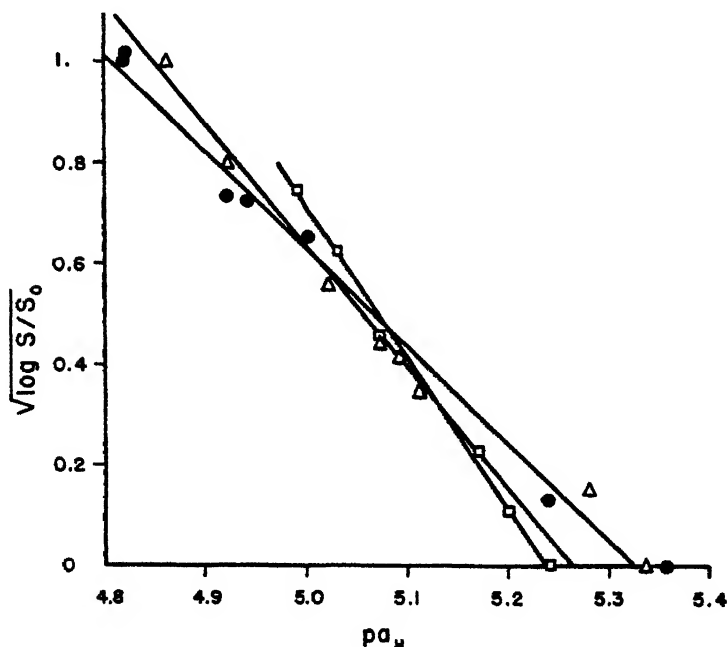


FIG. 1  
Solubility Curves after Gronwall

of the solubility determinations of Grönwall. Since the validity of (14) is limited to the acid side of  $pa_H^0$  we have only been concerned with the acid branch of the solubility curves. In drawing the lines more weight is given to the points corresponding to higher solubilities,

the analytical error being smaller here, but it must still be admitted that the slope is not very well determined. The solitary value for  $M_p$  in Table I should not, therefore, be stressed too much. That the molec-

TABLE I

	$\sqrt{M_p \frac{q}{2}}$	$M_p q$	(Grönwall) $q$	(Cannan <i>et al.</i> ) $q$	$M_p$
0.001	1.925	7.4	—	—	—
0.005	2.400	11.5	$3.18 \cdot 10^{-4}$	—	36000
0.010	2.876	16.6	—	$2.78 \cdot 10^{-4}$	(60000)

ular weight found by combining the results of Grönwall and those of Cannan, Palmer and Kibrick is so far off is rather surprising but may possibly be explained in part by the great differences in solubility found for different lactoglobulin preparations (7, 9). The deviation is, however, too great to be understood solely on this basis, and the value for  $q \cdot M_p$  at  $c_{NaCl} = 0.01$  is probably too high. It is true that  $q$  must rise with the sodium chloride concentration (2, 8) (and therefore the values of  $q$  found by Grönwall and by Cannan *et al.* do not agree) but the increase of  $q \cdot M_p$  from 11.5 to 16.6 for a doubling of  $c_{NaCl}$  is hardly possible. The question needs further investigation. We wish to call attention to the fact that the solubility minimum found by Grönwall is markedly on the alkaline side of the isoelectric point given by Cannan, Palmer and Kibrick and varies with the salt concentration. This may be due to a difference in the lactoglobulin preparation (see above) but may also be explained by assuming that the precipitate in the experiments of Grönwall was not "neutral" lactoglobulin but a salt of lactoglobulin. If this salt be of the composition [lactoglobulin,  $n X$ ] where  $X$  stands for a monovalent cation or anion we find, by a procedure analogous to that leading to (10),

$$\left( \frac{d \ln S}{d \ln a_H} \right)_{a_n} = \bar{i} - n \quad (18)$$

where  $n$  is negative if  $X$  is a cation, and positive if  $X$  is an anion. The activity of  $X$  must not vary with  $a_H$  or  $S$ . Introducing (11) and (14) gives

$$\frac{d \log S}{d p a_H} = M_p q (p a_H - p a_H^0) + n \quad (19)$$

and

$$\log (S/S_0) = M_p \frac{q}{2} (pa_H - pa_H^0)^2 + n(pa_H - pa_H^0), \quad (20)$$

where  $S_0$  is not identical with the minimum solubility. From (19) we obtain

$$n = -M_p q (pa_H^* - pa_H^0) \quad (21)$$

where  $pa_H^*$  is the reaction at the solubility minimum, and inserting into (21) the value of  $M_p q$  found by Cannan *et al.* ( $c_{KCl}=0.01$ ,  $M_p=40000$ ) we find that the displacement of the solubility minimum (Grönwall) may be tolerably well explained by putting  $n = -1$ . However, neither the discrepancy mentioned in connection with Table I nor the difference between the solubilities of different lactoglobulin preparations (Grönwall (7, 9)) is explained in this way. The question will be taken up in a later publication.

In some of the experiments of Grönwall the concentration of HCl added and, therefore, of protein hydrochloride, is of the same order of magnitude as the sodium chloride concentration.  $\bar{\varphi}_1$  (see equation (7)) might, therefore, be of significance here. The problem is hardly open to an exact treatment at the present moment and a detailed discussion shall not be attempted. It may just be pointed out that the well established fact that  $q$  for several proteins is practically independent of the protein concentration at moderately high salt concentrations and moderately low values of  $\bar{v}$  (10, 2, 8) gives a strong indication that  $\bar{\varphi}_1$  is a term of secondary importance.

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## The Urinary Excretion of *d*-Ribose in Man

No previous experiments have been reported in the literature regarding the urinary excretion of *d*-ribose after ingestion by humans. A few exploratory experiments have now been carried out and are being reported below in view of the fact that, for the present, no extension of this work is intended.

In the first experiment, one of us (H.M.W.) underwent a control period of 31 hours during which the average urinary sugar excretion was determined by the Hagedorn-Jensen-Hane method as 3.6 mg./cc. Then 20 g. of *d*-ribose were ingested and the total sugar excretion above the average was determined during the following 24 hrs., when the excretion had practically reverted to normal. The total excretion above normal (calculated as *d*-ribose) amounted to 1.56 g. or 7.8% of the ingested *d*-ribose. At this time another 20 g. of *d*-ribose was ingested and, during the following 24 hours, a total *d*-ribose excretion of 1.3 g. or 6.5% was observed; thereafter the sugar excretion was normal.

In the second experiment with another subject (U.V.S.), separate determinations were made of excreted total sugar and pentose, the difference representing hexose. While the total sugar was determined as before by means of the Hagedorn-Jensen-Hane method, pentose was determined colorimetrically by means of the Bial method as modified by Barrenscheen and Peham (1). During a 24-hour control period from 9 A. M. to 9 A. M. the following day, the pentose excretion was nil while the hexose excretion varied from 1.57 to 4.76 mg./cc., corresponding to a total of 4.45 g. of hexose (calculated as glucose). At the end of the control period, 25 g. of *d*-ribose was ingested and the first urine, 14 hours later, was found to contain 16.0 mg./cc. of pentose corresponding to 4.16 g. or 16.6% of the ingested *d*-ribose. At the same time the total sugar content was 17.35 mg./cc. indicating a normal hexose excretion. Subsequent pentose excretions were nil while hexose excretions continued on the normal level. In the same experiment, the blood sugar was determined by the same methods. During the control period the total blood sugar was found to vary between 67 and 152 mg./100 cc. while the blood pentose was nil. After ingestion the first blood sugar determinations were made just before the determination of the first urinary excretion (14 hours) and were found to be 100 mg. of hexose and 34 mg. of pentose (per 100 cc.). The next determination was made two hours later when the urinary pentose excretion was nil. At this time blood hexose was 65 mg./100 cc. and blood pentose 44 mg./100 cc. Two hours thereafter the blood pentose was nil.

The excreted pentose was isolated from urine as the  $\alpha$ -aniline-N-*d*-ribopyranoside-sodium sulfate complex followed by hydrolysis to give

crystalline *d*-ribose according to Berger *et al.* (2) and showed, after recrystallization, M.P. 74–78°C.,  $[\alpha]_D^{25} - 17.9^\circ$ ,  $C = 1\%$  in water. The product did not give a melting point depression with pure *d*-ribose, M.P. 84–87°C. but, in view of the somewhat low values of melting point and rotation, it was converted into the diphenylhydrazone. From the rotation  $[\alpha]_D^{25} - 9.5^\circ$ , and mixed melting point 147–149°C. with authentic *d*-ribose-diphenylhydrazone, M.P. 147–149°C., the excreted pentose was identified as *d*-ribose.

For comparison, another experiment was undertaken with the same subject (U. V. S.) ingesting *d*-xylose, the excretion of which after ingestion by men had previously been reported by McCance and Madders (3). During a 12-hour control period the total excreted sugar varied between 1.63 and 2.78 mg./cc. while the excreted pentose was nil. Then 20 g. of *d*-xylose was ingested and the first urine examined 10 hours later. The Hagedorn-Jensen-Hane method showed 17.5 mg. cc. total sugar while the Bial method showed 15.0 mg./cc. pentose. After a 4.5 hour interval there was a total of 15.5 mg. of total sugar and 7.3 mg. of pentose/cc. Later excretions showed normal total sugar values and no pentose. The total excreted pentose amounted to 46.3% which is almost three times as high as the largest excretion found for *d*-ribose and is somewhat higher than the 38% *d*-xylose excretion reported by McCance and Madders (3) after ingestion of 5 g. by healthy humans. These authors did not attempt to isolate the pentose excreted. We isolated *d*-xylose according to the general method of Greenwald (4) for the isolation of *d*-xyloketose. The isolated pentose was transformed into the *p*-bromophenylhydrazone with 20% overall yield, M.P. 122–124°C.,  $[\alpha]_D^{25} + 9.97^\circ$  after 24 hours,  $C = 3\%$  in ethanol, and its identity established with *p*-bromophenylhydrazone prepared from authentic *d*-xylose.

Without attaching undue weight to the low *d*-ribose excretion found in the few experiments reported above, it is interesting to note that Larson *et al.* (5) studied the "coefficient of absorption" from the intestine for *d*-ribose fed to rats by stomach tube. These authors found that the coefficient, defined as the average absorption in mg./100 g. body weight/hr., was 7 for *d*-ribose as compared with 178 for *d*-glucose, 77 for *d*-fructose, 34 for *d*-mannose, 28 for *l*-xylose, and 16 for *l*-arabinose as found by Cori (6). If the absorption coefficients in the human are comparable to those in the rat, which seems likely in view of the work of McCance and Madders (3), the low urinary *d*-ribose excretion would appear to be due to its poor absorption from the intestine.

#### ACKNOWLEDGMENT

We wish to express our thanks to Mr. Edward Wenis and Mr. Fred Leonard for carrying out numerous sugar determinations.

## SUMMARY

In an exploratory study the urinary excretion in man after ingesting *d*-ribose was found to be 6.5 and 7.8% in one subject and 16.6% in another.

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### Interaction of Serum Albumin, Free and Esterified Oleic Acid and Lipase in Relation to Cultivation of the Tubercle Bacillus

We have recently reported that water-soluble esters of long chain fatty acids facilitate submerged growth of the tubercle bacillus in liquid media; on the other hand, serum albumin is necessary for growth of small inocula of this organism (1, 2, 3). Attention has been centered on the polyoxyethylene derivative of the sorbitan ester of oleic acid (commercial "Tween 80," Atlas) and on bovine serum albumin (commercial Fraction V, Armour). The albumin has been shown to function predominantly as a protective rather than a nutritive growth factor (4). Since this protein was found to antagonize the bacteriostatic effect of a variety of substances, including fatty acids, the role of oleic acid in Tween was investigated. This led to the following evidence that serum albumin has the apparently unique property of tightly binding oleic acid.

An analytical method was developed for measuring small quantities of fatty acid in the presence of Tween, involving ether extraction of

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an acidified solution saturated with  $\text{Na}_2\text{SO}_4$ . A number of batches of Tween 80 were thereby shown to contain 0.6% by weight of unesterified fatty acid. It was found possible to free Tween 80 of this fatty acid by a similar method.

This purified Tween permitted accurate definition of the role of albumin. The medium containing 0.05% of untreated Tween would not permit growth of small inocula ( $10^{-7}$  mg. moist weight of human tubercle bacillus H37Rv in 6 ml.) unless albumin was added. The substitution of the purified Tween, however, permitted growth of  $10^{-7}$  mg. without albumin. Since small inocula were inhibited, in a medium containing purified Tween, by addition of 1 part per million of oleic acid, and large inocula ( $10^{-2}$  mg.) by 10 p.p.m., it is clear that the toxicity of the medium containing untreated Tween can be accounted for by its content of free fatty acid (3 p.p.m.).

Both bacteriological and more direct evidence of interaction between albumin and oleic acid have been obtained. The addition of varying amounts of oleic acid to media containing 0.05–1% albumin indicated that the protein binds approximately 2% of its weight of the fatty acid sufficiently completely to permit growth of small inocula of tubercle bacilli. Albumin is capable of binding even greater amounts of oleic acid (approximately 3%, or 9 molecules/molecule of protein), as estimated by criteria which measure less complete binding: extinction of the opalescence of a neutralized solution of oleic acid, and inhibition of its hemolytic effect, upon addition of albumin. Since a variety of proteins other than serum albumin had been found ineffective in promoting the growth of small inocula (1, 2), some of these were tested for their ability to bind oleic acid, using opalescence and hemolysis as criteria. Gelatin, protamine, papain and ovalbumin were without effect;  $\beta$ -lactoglobulin bound approximately half as much as serum albumin, but not tightly enough to contribute to the growth of small inocula. (Cf. (5) for a looser interaction of serum albumin with short-chain fatty acids).

The "albumin" was found to exert an undesirable as well as a beneficial effect in the medium. Bacteriological evidence of instability of the medium suggested that the commercial albumin (Fraction V) contains a small amount of lipase which in 1–2 weeks hydrolyzes enough Tween to exceed the binding capacity of the albumin. The presence of lipase was confirmed chemically. The lipase can be destroyed by heating a neutral solution of albumin to 56°C. for 30

minutes, or inhibited by addition to the medium of 0.01% NaF. In addition, lipase can be avoided by using the purer commercial crystalline bovine serum albumin. If the lipase activity is eliminated by any of these means, it is possible to obtain visible growth regularly from one further ten-fold serial dilution ( $10^{-8}$  mg. moist weight, or 2-3 cells) than was previously possible.

The effect of lipase on Tween furnished the explanation for the curious observation that horse serum (but not human or bovine serum) in concentrations of 5-20% prevented growth in this medium, whereas 40% permitted growth. It was found that horse serum contains more lipase than human serum; it is known (6) to have less albumin. Apparently the 40% horse serum permitted growth by binding all the fatty acid available by hydrolysis. This experience emphasizes the importance in diagnostic work of inhibiting or counteracting the effect of lipase which may be added with the inoculum.

In summary: although Tween 80 promotes the growth of tubercle bacilli, it inhibits small inocula. This inhibition is produced by its content of unesterified fatty acid, which can be extracted. Serum albumin neutralizes the inhibition through a specific capacity to bind tightly the fatty acid, but it also contains a trace of lipase. Growth from truly minimal inocula (2-3 cells) is possible only when hydrolysis of the Tween by this lipase is prevented.

The details of these experiments are to be published.

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## Book Reviews

**On the Structure of the Protein Molecule.** Second Edition by N. TROENSEGAARD. Einar Munksgaard, Copenhagen and Humphrey Milford, Oxford University Press. London, 1944.

In the preface to this book, the author states that "the first edition of the present volume, which was published in German, aroused so much interest that the author felt encouraged to issue a second edition, revised and enlarged, and this time in English." The purpose of the work is to reiterate the hypothesis that proteins are composed largely of heterocyclic ring systems which are readily split by acid, alkali and enzymes. According to this view, the amino acids which arise through the action of these agents are secondary reaction products, and do not exist as such in proteins. This theory was first offered by Troensegaard in 1920 and in support of it, there were reported, during the period 1920-1930, a series of experiments in which the "drastic" methods which were known to yield amino acids from proteins were avoided. Instead, proteins such as gliadin, gelatin, casein and serum globulins were carefully desiccated and then acetylated at elevated temperatures either with acetic anhydride after treatment with methanol-KOH, or in glacial acetic acid solution with acetyl chloride. The "acetyl-proteins" thus obtained were then subjected to reduction by means of sodium and amyl alcohol at 115-155°C. This was followed by hydrolysis and separation, often by distillation at high temperatures, of the resulting mixture into a number of fractions. Although the isolation of a large number of substances is reported, the yield in each case was apparently very small and the characterization, in most cases, inadequate. The presence, among these products, of substances claimed to be derivatives of piperidine, pyrrolidine and piperazine, is taken to provide experimental evidence for Troensegaard's theory.

It is difficult to accept the claim that a series of manipulations of this kind represents mild treatment of a protein and one which is likely to preserve intact the characteristic linkages of proteins. Rather, there is evidence from the work of Dakin, Bergmann and Karrer (which Troensegaard does not mention) to show that the treatment of amino acid derivatives with reagents such as acetic anhydride may give rise to a variety of heterocyclic ring systems. Furthermore, although the author cites, in support of his theory, the isolation, by Dakin and others, of diketopiperazines from protein hydrolyzates, he does not mention the extensive studies during the 1930's on the resistance of diketopiperazines to proteolytic enzymes, nor does he refer to the well-known capacity of certain peptides to form such ring structures.

In perusing this work one cannot fail to be dismayed by numerous remarkable statements such as that on p. 39: "That an oxygen atom can be the connecting link between cyclic systems is shown by the circumstance that thyroxine can be split off from casein treated with iodine. . . . This hormone therefore has its origin in a prosthetic group of the casein, the alanine residue of the group presumably being anchored in a diketopiperazine ring."

With the argument of the author that new chemical methods are needed for the study of protein structure, no student of the subject can disagree. Such methods may well emerge from experimental work based on the most tenuous of working hypotheses. It seems best, however, to suspend discussion of such a hypothesis if, after due time, it has failed to win acceptable experimental support.

JOSEPH S. FRUTON, *New Haven, Conn.*

**Statistical Methods Applied to Experiments in Agriculture and Biology.** By GEORGE W. SNEDECOR, Director of the Statistical Laboratory of Iowa State College and Head of the Statistical Section of the Iowa Agricultural Experiment Station. The Iowa State College Press, Ames, Iowa. 1946, xvi+485 pp. Price \$4.50.

Snedecor's leadership in statistical work at Iowa and in the country at large is well known; his writings are familiar. The book under review has come to its fourth edition, and with it to a large degree of rewriting. The book is descriptive, not analytical; it tells what to do rather than why one should do it, and for that reason it is well that the author emphasises that the examples, which are many, both worked and unworked, are an essential part of his presentation. Thus chi-square is defined and applied very early without any mathematical treatment which might show the basic rationale of the method. Although the results of mathematical statistics are thus freely used without giving their background, the author does try to make them reasonable to the limited extent that verbal explanations can do so. This may be pedagogically the best thing to do in view of the necessary use of statistics by persons poorly equipped mathematically. Certainly the emphasis laid upon the conditions in which the methods have validity and upon the conduct of experiment so as to incorporate in the data the information which is desired are elements even more necessary for most working statisticians than the mathematical derivations of the methods.

It is too much to expect that in so long a book, carefully though it be written, there should not be statements to which one might take exception. Thus (p. 7) "The possession of an attribute by an individual is a fact that may be certainly determined" seems somewhat exaggerated; corresponding to errors of measurement, there are oftentimes indeterminacies of classification. And I do not like the discussion (p. 38) of the reason for dividing the sum of squares in a sample of  $n$  by  $n-1$  instead of by  $n$  to get the estimate of the variance of the population; for I cannot persuade myself that "It lies in the improbability of drawing representatives of the population extremes in small samples. The range of the sample is likely to be much less than that of the population. The sample mean square deviation (variance) would likely fall short of the parameter if the division were by  $n$ ",—but I may be wrong, and it might take a real mathematical discussion to decide. Another item I do not like is the discussion (p. 50) of asymmetry of the distribution of weights of individuals. On p. 42 and on p. 62 we have the formulas

$$\pm t = (\bar{x} - m)/s_x \quad \text{and} \quad t = (\bar{x} - m)/s_y$$

which mean the same thing with a difference in notation that may well be confusing. But I will not persist in citing what seem to me to be infelicities, as I am sure that, should I write a five hundred page book on statistics, there would be in it as many

book. I should subsequently esteem doubtful as I could point out in Snedecor's

The analysis of variance may be said to be the most widespread method, the most pervasive idea in the book. This is a relatively new method and Snedecor's attachment to it is well known. Its applications are varied and unless treated in detail are likely to bother those who would learn the method and use it. I know of no better exposition than Snedecor's.

In the treatment of the 4-fold table I do not find the so-called "exact method" expounded, though there is a reference to Yates's article in which the method was first developed in print. Incidentally, it should be remarked that there are listed a good many references which will enable the student to pursue one subject or another well beyond the exposition in the text. It should be remarked that Snedecor's Statistical Methods is really a presentation of the Pearson-Fisher analysis, the analysis of the English biometric school; the names of the continental statisticians Laplace, Levis, Thiele, Charlier, Wicksell do not occur in the index, nor for his purposes need they.

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**Physical Methods of Organic Chemistry.** Vol. II. Editor, ARNOLD WEISSBERGER. Eastman Kodak Company Interscience Publishers, Inc., New York, N. Y., 1946. VII+640 pp. Price \$8.50.

The title of this and its companion volume is misleading. Physical methods described in the twenty-six articles are really treated as applicable to any branch of chemistry which is an entirely justifiable approach. In fact the organic chemist will be disappointed to find that the physical methods used in his work are not far enough up the scale to be included. What he finds in the two volumes, however, should give him a better grasp of the basic science necessary for measuring the properties of matter with considerable accuracy. A better title might have been "Physical Methods for the Physical Chemist."

In the first three articles of this second volume, namely: Spectroscopy and Spectrophotometry (85 pp). W. West, Colorimetry; Photometric Analysis, and Fluorimetry (45 pp.) W. West, Polarimetry (120 pp). W. Heller, the authors adhere closely to the physics of the physical methods and do a splendid job. These are classical subjects that have reached a high level of perfection. The authors waste none of their allotted space on photographs of instruments currently available but rather go into the optical principles involved. Organic chemists might find the article on polarimetry unsuited to their purposes unless they are particularly interested in the many alternative devices or have to make a choice in purchase of a new instrument. A more thorough treatment of the errors in spectrophotometry due to scattered light might have been given since this is of such great importance at wave lengths shorter than 2500 Å. Adequate emphasis is placed on visual colorimetry. A more chemical approach is adopted in the four articles; Conductometry (40 pp). T. Shedlovsky; Polarography (97 pp). O. H. Müller; Potentiometry (65 pp). L. Michaelis; Determination of Magnetic Susceptibility (35 pp). L. Michaelis. The basic chemistry of potentiometry and of oxidation-reduction potential measurement is so beautifully covered by Michaelis as to justify the purchase of the book by an organic chemist. Precautions and pitfalls are placed in their correct perspective. The article on magnetic suscepti-

bility is weaker and might better have been treated from a physical point of view. It neither adequately goes into the principles involved nor presents the merits of alternative methods. The article on conductometry is too condensed for adequate treatment and is more of a discussion of theory than of method. Electrophoresis, which could better have been the subject for the whole section, is covered in six pages. An adequate treatment is given of ionization constants of weak electrolytes which is particularly applicable to organic chemistry.

The reviewer was completely ignorant about polarography but after reading Müller's section feels that he can make profitable use of the methods. Polarography perhaps started as a child of ill repute without too clear cut an idea as to what was being measured. Twenty years of research, however, have partially clarified this situation and the subject now rests upon an apparently firmer basis.

Volume II is rounded off by three short articles, namely: Determination of Dipole moments (20 pp). C. P. Smyth; Determination of Radioactivity (40 pp). W. F. Bale and J. F. Bonner; Mass Spectrometry (24 pp). D. W. Stewart. All of these bear the mark of having been thrown off in as short time as possible and the last two are so inadequate as to be misleading. One might have expected to find a discussion of the principles involved in the Consolidated Engineering Corp., mass spectrometer rather than a photograph of its instrument panels. The important subject of the nature of ionization by electron impact in the ion source is hardly mentioned. Statistical fluctuations in measurement of radioactivity with Geiger-Müller counters are treated according to the usual theory, of errors rather than by use of Poisson's Formula. The article on radioactivity, however, does have the merit of being the only one in the two volumes that presents electronic circuits in working detail. Organic chemistry in the future will probably draw more heavily on isotopic trace techniques than on polarimetry.

Instrument manufacturers rather like to leave things a bit mysterious and reduce observations to orderly punching of switches. In these volumes the basic simplicity of the various measuring devices is emphasized. Necessary compromises of commercial instruments can often be corrected if the principles involved are thoroughly understood. While the organic chemist will not find here a modern Houben-Weyl's "Arbeitsmethoden" he should not fail to make use of the labors of so many men in giving the principles of many physical measurements in a reasonable space.

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**Immuno-Catalysis.** By M. G. SEVAG, Ph.D., Assistant Professor of Biochemistry in Bacteriology, Department of Bacteriology, University of Pennsylvania School of Medicine, Philadelphia. Charles C. Thomas, Publisher, Springfield, Illinois, 1945. viii 272 pp. Price \$4.50.

In his book "Immuno-Catalysis," Dr. M. G. Sevag shows that there exists a striking parallelity between immune and enzymatic reactions. This book should be brought to the attention of all biochemists who are interested not only in the accumulation of new facts important to biochemistry but also in the application of these findings to other fields of research. It should also provide stimulating reading for bacteriologists and serologists looking for new tools of research.

The first part of the book, entitled "Antigens as Biocatalysts" deals with the formation of antibodies and their chemical properties as well as with chemical aspects of

catalytic procedures; it concludes with a discussion of the mechanism of antibody formation. In the second part, "Antibody as a Specific Enzyme Inhibitor," immune reactions are compared with enzyme reactions and the formation of specific inhibitors in enzyme reactions is discussed in detail and illustrated with many examples. Part III, "Anti-Enzyme Immunity," deals with the problem of anti-enzymes and discusses the function of antibodies as that of enzyme inhibitors. It contains a detailed report on the formation of antibodies against various purified enzymes and the inhibitory action of immune sera formed. Part IV deals with "Immunity Against Bacterial Enzymes." The enzymatic activity of certain bacterial toxins is discussed, and the formation of antibodies against bacterial toxins is evaluated. In Part V, "The Problem of Antibody Formation Against Respiratory Enzymes" is treated.

The fact that Dr. Sevag consulted more than a thousand original papers testifies to the thoroughness of his approach. Besides having collected interesting experimental data which were scattered throughout the international scientific literature, and having correlated them with immunological facts, the author presents these data in a logical manner. He discusses many fundamental facts of enzyme chemistry, as far as this is necessary for an understanding of his treatise. This enables the reader who is not very familiar with biochemistry to follow the author's trend of thought without consulting other sources. Not all enzyme chemists might be willing to accept Dr. Sevag's suggestion concerning a wider definition of an enzyme. However, his critical analysis of the similarity of enzyme functions and those of antigens seems logical and stimulating for an understanding of the chemical mechanisms of immune reactions; he further strengthens his concept by considering antibodies as enzyme inhibitors. An attractive feature of his presentation is that he frankly admits that in some instances the available evidence for a close parallelity between immune reactions and enzyme reactions is not yet conclusive and that more experimental data are needed.

In a preface to the book, Dr. Stuart Mudd stresses the importance of cooperation among various branches of science and states that "Teachers and investigators dealing with biochemistry, with bacteriology and immunity and with the mechanisms of infectious disease will, I believe, find in this informative book an expanded horizon and a challenge to further investigations." This reviewer feels that the content of Dr. Sevag's work could not be better characterized.

KURT SALOMON, Rochester, N.Y.

# The Effect of Varying the Conditions of Hydrolyzing Casein with Sulfuric Acid Upon the Optical Rotation and the Amino and Ammonia Nitrogen Content of the Hydrolyzates \*

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## INTRODUCTION

The autoclave may be used to shorten the time or lower the concentration of acid required to hydrolyze a protein, but continuing the heating longer than necessary to complete the hydrolysis induces racemization (1) and a decrease in amino nitrogen (1-3).

Previous studies from this laboratory have involved the use of zein (1, 4). In extending these to determine the degree of racemization and destruction of specific amino acids liberated under practical conditions of hydrolysis, it seemed preferable to turn to casein because the latter is soluble in acid and more readily available commercially. In the isolation of amino acids or in preparation of hydrolyzates for use in the diet, sulfuric acid possesses the advantage of being more readily removed after the hydrolysis is complete; high concentrations of protein are obviously more practical. Unfortunately, most of the tests which have been made hitherto with casein have involved the use of hydrochloric acid and relatively low concentrations of the protein (5). Before proceeding to isolation studies, therefore, preliminary tests were made to determine the influence of variations in temperature, time of heating and concentration of sulfuric acid upon the optical rotation and the amino and ammonia nitrogen contents of the hydrolyzate as a whole. These basic tests are summarized below, together with com-

\* The experimental data in this paper are taken from a dissertation submitted by Arnold H. Schein in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.



parisons of the degrees of hydrolysis effected in tests with sulfuric acid and hydrochloric acid solutions of low normality conducted simultaneously, and observations on the influence of ammonia upon the estimation of amino nitrogen.

### EXPERIMENTAL

The hydrolytic procedures were essentially those of Borchers and Berg (1). The commercial acid-precipitated casein contained moisture, 8.0%; ash, 2.5%; ether-extractable material, 1.2%; and nitrogen, 13.39%. When calculated on an ash-, fat- and moisture-free basis, the nitrogen content becomes 15.1%, that of highly purified casein, 15.5–15.6% (6). Hence, 1.164 g. of the commercial casein were used in each test as the equivalent of 1 g. of the pure protein. With this were mixed 0.6 g. of acid-washed charcoal (to clarify the hydrolyzate) and 10 cc. of an aqueous  $H_2SO_4$  solution, 8, 25 or 33% by volume (3.0, 9.4 or 12.6 *N*, by titration). The time and mode of heating are indicated in the protocols.

The hydrolyzates were filtered while still hot, the residues washed on the filter with hot water, and the combined filtrates cooled and diluted to exactly 25 cc. Optical rotation was read in a Schmidt-Haensch polariscope with a 2 dm. tube and a sodium vapor lamp, at room temperature. Correction for variations in the latter again proved to be unnecessary (1, 7). Total nitrogen was determined by the macro Kjeldahl procedure; ammonia by diluting an aliquot (10:150) of the filtrate, making it just alkaline to phenolphthalein, then adding 1 cc. of a 5% solution of sodium carbonate, distilling and titrating the distillate; analysis of the ammonia-free residue for amino nitrogen by the macro method of Van Slyke, using a reaction period of 4 minutes.

Filtrates from completely hydrolyzed casein contained 148 mg. of total nitrogen, residues 7–8 mg. The filtrates showed a maximal rotation of  $+1.00^\circ \pm 0.05^\circ$ . Winnick and Greenberg record the rotation of a filtered casein hydrolyzate (prepared by boiling 12 g. of casein under the reflux for 33 hours with 300 cc. of 1:5 HCl) as  $\alpha_N^{25} = +88^\circ$  (7). Since  $\alpha_N$  is the rotation of 1 dm. of solution containing 1 g. of total nitrogen/cc., our maximal rotation is equivalent to  $\alpha_N^{25} = +84.5^\circ \pm 4.2^\circ$ . To facilitate comparison, rotations observed on the few solutions with less than the usual 5.92 mg. of nitrogen/cc. were "corrected" to compensate for the deviation.

The normal percentages of total nitrogen found as ammonia and amino nitrogen were  $10.8 \pm 0.5$  and  $69.8 \pm 0.5$ . The literature records 9.8 (8), 10.3 (11) and 10.2% (9) for the former; 69.1 (10) and 70.0% (11) for the latter.

That ammonia affects the measurement of amino nitrogen (12, 13) was confirmed. In 4 minutes at 30°C. 0.2 and 0.4 *N* solutions of ammonium sulfate liberated as much as 40% of their nitrogen, whether tested before or after admixture with amino acids or protein hydrolyzates.

The results of the tests with 25% sulfuric acid are presented in Fig. 1. With the 33% acid, hydrolysis was more rapid (complete in 8 hours under the reflux, 1 hour at 140°C.), but subsequent changes were not appreciably so; hence the data are omitted. In none of the tests with

the 8% sulfuric acid solution were the maximal values for amino nitrogen or rotation as high, but the ammonia found was about the same. Ammonia is readily produced from amide groups, even with dilute acids (14). Its increase upon prolonging the period of heating with 25 or 33% acid is accompanied by a decrease in amino nitrogen

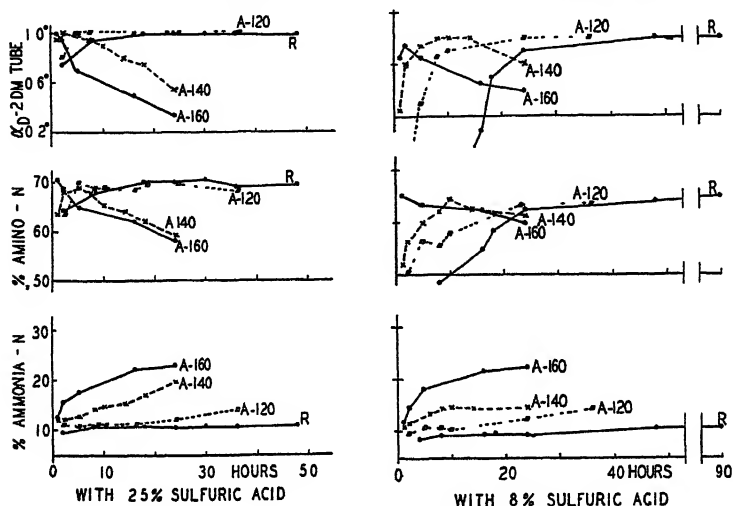


FIG. 1

Changes in Optical Rotation and Amino and Ammonia Nitrogen Content on Heating Casein with 25% (by Volume) and 8% (by Volume) Aqueous Sulfuric Acid Solutions

The curves connect points representing analyses obtained on hydrolyzates prepared by boiling under the reflux (R) or by heating in the autoclave (A) at 120°, 140° or 160°C. In the latter, the period was timed to include the period of temperature rise from the initial 100°C. (about 1 hr. for an increment of 80°C.), but not the period of cooling. Most of the values for  $\alpha_D$  represent uncorrected readings in a 2 dm. tube. On a few incompletely hydrolyzed samples, corrections were necessary for correlation with the other data (see text). With the 8% acid, the  $\alpha_D$  was  $-1.91^\circ$  after 4 hrs. of boiling under the reflux,  $-0.65^\circ$  after 8 and  $+0.10^\circ$  after 16 hrs. After 4 hrs. under the reflux the amino nitrogen was 35.5%. After 2 hrs. of heating in the autoclave at 120°C. the  $\alpha_D$  was  $-0.50^\circ$ .

(15), from which it may originate, at least in part. This relationship was less marked in the tests with the 8% acid, possibly because amino nitrogen was still being liberated from the incompletely hydrolyzed casein. The temperature seemed to exert a greater influence than did the concentration of acid.

In confirmation of earlier conclusions (16), hydrolysis under the reflux with 3 *N* HCl solution was found to be about 98% complete in 17 hours; with 3 *N* H<sub>2</sub>SO<sub>4</sub> solution, only 83% complete in 17 hours and 93% in 90. Results of similar tests with mixtures heated in the autoclave in sealed Pyrex tubes (Table I) indicate incomplete hydrolysis

TABLE I

*Nitrogen Distribution in Hydrolyzates Prepared by Heating 1.5 g. of Casein with 50 cc. of 3 N HCl and H<sub>2</sub>SO<sub>4</sub> in Sealed Pyrex Tubes*

	Heated 3 hrs. at 127° C.		Heated 1½ hrs. at 150° C.	
	with HCl	with H <sub>2</sub> SO <sub>4</sub>	with HCl	with H <sub>2</sub> SO <sub>4</sub>
Ammonia Nitrogen <sup>+</sup>	10.0	9.3	10.7	11.0
Amino Nitrogen (determined after removing ammonia)*	62.2	47.4	70.5	59.6
Amino Nitrogen (determined without removing ammonia) <sup>+</sup>	65.2	52.2	76.0	64.5

\* Expressed as *per cent* (g./100 g.) of total nitrogen.

in 3 hours at 127°C. with both acids, complete hydrolysis in 1½ hours at 150°C. only with the hydrochloric.

Two earlier reports record complete hydrolysis at 125°C. with 3 *N* HCl in 3 hours (2, 10), a third incomplete hydrolysis even after 6 hours (17). Failure to remove ammonia in the third test quite likely accounts for the high amino nitrogen value recorded as indicative of complete hydrolysis.

### SUMMARY

Tests of the hydrolysis of casein, in practical concentration approximating 1 g./10 cc. of an aqueous solution of sulfuric acid, 8, 25 or 33% by volume, showed that the 25 and 33% acids yielded hydrolyzates with the same maximal values for optical rotation and amino nitrogen concentration, but that the highest values obtained with the 8% acid were definitely submaximal. Beyond the slight decrease in time required for complete hydrolysis, the 33% acid afforded no advantage over the 25% acid. Except for small initial differences, the ammonia production was the same with all three concentrations of the acid.

Prolonging the heating under the reflux to 90 hours with the 8% acid or to 48 hours with the 25 and 33% acids did not alter the maximal optical rotation or the maximal amino nitrogen attained earlier, nor did it increase the ammonia content. In the autoclave at 120°C. the content of ammonia increased slightly in 36 hours. Prolonging the heating at 140°C. and 160°C. produced rapid increases in the concentration of ammonia and rapid decreases in amino nitrogen and optical rotation. The increase in ammonia appeared to occur largely at the expense of the amino nitrogen. Racemization was indicated by the proportionately greater decrease from maximum in optical rotation than in amino nitrogen. The temperature seemed to exert an appreciably greater influence than the acid concentration in inducing the changes noted during the periods of prolonged heating.

Direct comparisons with 3 *N* hydrochloric acid and 3 *N* sulfuric acid showed more rapid hydrolysis of casein in every case by the former acid. Despite the smaller concentration of casein employed in these comparative tests, none of the hydrolyzates prepared with the sulfuric acid showed the maximal concentration of amino nitrogen associated with complete hydrolysis.

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# The Effect of Varying the Conditions of Heating Casein with Sulfuric Acid Upon the Yields and Optical Rotations of the Basic Amino Acids \*

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## INTRODUCTION

Acid hydrolyzates of zein (1) and casein (2) prepared at temperatures which prevail under the reflux showed no appreciable change from the maximal amino nitrogen content and the optical rotation associated with complete hydrolysis, even when the heating was continued for 48 hours; but when the hydrolyzates were prepared in the autoclave at elevated temperatures, prolongation of the period of heating produced decreases from both maxima which became more marked as the time was extended and underwent acceleration as the temperature was raised. Fairly drastic conditions of heating yielded hydrolyzates deficient in threonine; still more severe conditions produced deficiencies not fully remediable by adding threonine alone (3). Chemical assays suggested that the threonine was not merely racemized or epimerized, but was actually largely destroyed (3).

The purpose of this paper is to present observations made on histidine, arginine and lysine in a study in which these amino acids were isolated as derivatives from a series of variously prepared hydrolyzates of casein and subsequently converted into hydrochlorides suitable for polariscopic assay. Comparisons of the yields of the derivatives isolated afforded a means of measuring approximately the extent of destruction imposed by each of the several conditions of heating em-

\* The experimental data in this paper are taken from a dissertation submitted by Arnold H. Schein in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

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ployed, and comparisons of the specific rotations of the hydrochlorides a means of estimating the degree of racemization. Histidine, arginine and lysine were chosen for study, chiefly because the methods of estimating them gravimetrically are among the best.

Some years ago Gortner and Holm (4) observed that, during a 6-week period of heating fibrin under the reflux with 20% HCl, a gradual destruction of amino nitrogen occurred after the first 48 hours. Inasmuch as the phosphotungstate fractions showed little or no change in content of nitrogen, the deamination was assumed to have occurred at the expense of the monoamino acids alone. The degradation of the monoamino acid, tryptophan, commonly observed during acid hydrolysis (5) probably does not contribute appreciably to the formation of ammonia (4). Deamination and dismutation of cystine occur when the heating is prolonged (6), but would be relatively inconsequential in the case of casein which contains so little cystine. At least at elevated temperatures, destruction of threonine (3) and serine (7) takes place. In our preliminary tests on autoclaved hydrolyzates of casein (2) increases in ammonia content on prolonged heating differed too widely from losses of  $\alpha$ -amino nitrogen to justify assuming that the ammonia was produced solely from the monoamino acids.

The three basic amino acids are all known to undergo racemization when heated separately in acid solution in the autoclave (8-10). In the case of arginine (9), cleavage of the molecule may possibly have been responsible for the small amount recoverable. We know of no previous attempt to determine the relative degrees of destruction and racemization induced in the three simultaneously in variously prepared acid hydrolyzates.

## EXPERIMENTAL

The casein was from the lot used previously (2), but the samples were 100 times as large (116.4 g.). They were hydrolyzed in 3-liter round bottom flasks with 1 liter of 25% (by volume) sulfuric acid. The time and temperature were varied to yield a series of hydrolyzates having an appreciable range in optical activity and nitrogen distribution.

*Isolation of the Amino Acids.* A small aliquot of each hydrolyzate was removed for polariscopic assay and amino nitrogen estimation (2). From the remainder the sulfate ion was precipitated with barium. The filtrate and washings were concentrated *in vacuo* to crystallize out, and largely remove, the less soluble amino acids and thus prevent their subsequent interference in some of the proposed isolation procedures. Exactly half of the filtrate and washings from these acids was used for the isolation of arginine, the other half for the isolation of histidine and lysine. Arginine was precipitated as the difluoride and converted to, and weighed as, the monofluoride (11). Histidine was precipitated with silver nitrate, first at pH 13, then at pH 7.2-7.4 (12). It was estimated as the nitranilate (13). Attempts to isolate

arginine as the monoflavanate from the filtrate and washings obtained in the second precipitation of the histidine produced lower yields than the more direct procedure. The filtrate and washings from the first histidine precipitate were used for the separation of lysine, initially as the phosphotungstate, subsequently as the picrate.

Yields equivalent to 3.74 g. of arginine and 2.24 g. of histidine per 100 g. of protein (15.6 g. of nitrogen) in the 116.4 g. of casein were attained. When the usual correction was applied to compensate for the solubility of the lysine phosphotungstate (14), the lysine equivalent became 6.42 g. Calculations on the same basis from isolation data in the literature give 3.88 (15) and 3.73 g. (11) of arginine; 2.40 g. of histidine (10); and 6.29 (13) and 5.81 g. (16) of lysine. Table I records the yields of derivatives from the various hydrolyzates. The amounts of arginine monoflavanate isolated from aliquots of the same hydrolyzate and from separately but similarly prepared hydrolyzates usually differed by less than 2%. Yields of histidine nitrilate from similarly prepared hydrolyzates usually agreed within 2.5%, those of lysine picrate within 2%.

The derivatives isolable after 18 hours at 120°C. and 5 hours at 140°C. approximated in quantity those isolable from the 24-hour hydrolyzates prepared under the reflux, but longer heating in the autoclave lowered the yields. Apparently all three of the amino acids underwent some destruction, arginine the most rapidly, lysine the least, and histidine at an intermediate rate. Appreciable coprecipitation of peptides seems contraindicated by the invariably lower weights of precipitate obtained in the periods which were too short for complete hydrolysis. The presence of ornithine could have interfered with the lysine determination (17, 18), but its picrate decomposes around 208°C. (18) and the lysine picrate preparations all exploded at 250°C. or above. This seems to preclude any appreciable contamination with ornithine. The influence of other degradation products is uncertain. Modification of the solubilities of the derivatives seems a more likely possibility than coprecipitation with them.

*Preparation and Optical Rotation of the Hydrochlorides.* Since all three of the amino acid derivatives isolated initially were highly pigmented and, therefore, unsuitable for polariscopic assay, it was necessary to convert them into products which could be compared more effectively. The colorless, water-soluble hydrochlorides described below were chosen for this purpose. The arginine monohydrochloride was prepared from the flavanate as directed by Cox (19). The histidine nitrilate was decomposed by suspending it in water, heating it rapidly to boiling, adding barium chloride slightly in excess of the amount reacting to form the heavy granular barium nitrilate and then cooling the mixture in an ice bath. After a few hours the precipi-



TABLE I

*The Influence of Various Conditions of Heating with 25% H<sub>2</sub>SO<sub>4</sub> on the Yields of Arginine, Histidine and Lysine from Casein*

Hydrolyzate No.	Observed Rotation of the Hydrolyzate	Ratio of Amino N to Total N	Derivative Isolated <sup>^</sup>		
			Arginine Flavianate	Histidine Nitrilanate	Lysine Picrate
	degrees	per cent	g.	g.	g.
R-24-1	+1.0	70.0	2.495 2.524	2.805	7.720
R-24-2			2.464	2.740	
R-24-3				2.782	7.620
A-120-2.5	+0.95	69.9	1.627 1.566	2.198	
A-120-4	+0.98	70.5	1.881 1.704	2.108	7.372
A-120-10	+1.0	69.0	2.149 2.228	2.758	7.238
A-120-18	+1.0	69.5	2.550 2.530	2.771	7.792
A-120-36	+1.0	66.5	2.258 2.283	2.846	7.979
A-140-1	+0.95	63.5	1.694 1.654	2.466	7.485
A-140-5	+1.0	69.0	2.463 2.449	2.880	7.402
A-140-14	+0.85	64.0	1.972 1.988	2.420	7.112
A-140-18	+0.75	62.0	1.904 1.898	2.119	6.804
A-140-24	+0.35	59.0	1.332 1.305	2.355	7.291
A-160-24	+0.35	59.0	1.471 1.496	2.237	7.237

<sup>^</sup> R indicates that the hydrolyzate was prepared by heating under the reflux, A by heating in the autoclave; the first figure which follows R indicates hours of heating; the second indicates the number of the sample. The first figure which follows A indicates the autoclave temperature, the second the hours of heating.

\*\* The weights given represent isolations, uncorrected except for the small aliquot removed for estimating nitrogen partition. Each figure for arginine flavianate represents the weight of precipitate from one-fourth of the hydrolyzate, each figure for histidine nitrilanate and for lysine picrate the weights of these derivatives from the remaining half.

tate was removed by filtration, the filtrate was freed of barium ion and the histidine converted through the monohydrochloride to the optically more active methyl ester dihydrochloride, essentially as outlined by Conrad and Berg (10). Rapid heating and cooling during the process of decomposing histidine nitrilate apparently curtails the formation of degradation products enough to prevent the copious amorphous precipitation which otherwise occurs when the aniline is added to convert the histidine to its monohydrochloride. The lysine picrate was recrystallized from water twice, after which it usually exploded sharply near 260°C. (uncorr.); it was then converted to the dihydrochloride in the usual way.

Data pertaining to the hydrochlorides are recorded in Table II. The extremely variable, and far from quantitative, yields reflect losses involved in recrystallizations made to insure purity. Analyses showed that the samples of arginine monohydrochloride contained 26.2–26.6% total nitrogen; those of histidine methyl ester dihydrochloride 17.2–17.4%; and those of lysine dihydrochloride 12.6–12.8%. Calculated values are 26.6, 17.30 and 12.79%, respectively. The melting points of the unracemized samples agreed well with those recorded in the literature, particularly when probable differences in rate of heating were considered. The melting points of the more strongly racemized samples of arginine monohydrochloride and lysine dihydrochloride were markedly lower, but this was not true of the racemized histidine methyl ester dihydrochlorides. The specific rotations of the unracemized hydrochlorides agreed well with the values recorded in the literature, + 21.22° for arginine monohydrochloride (20), + 9.45–+ 9.80° for histidine methyl ester dihydrochloride (10) and + 15.63° for lysine dihydrochloride (21). In so far as possible, the same conditions were employed in the measurement as in the reference cited. At 140°C. histidine was apparently racemized more rapidly than either lysine or arginine; in 24 hours at 160°C. the racemization of all three was complete.

*Influence of Racemization on the Yield of the Derivative Precipitated and the Optical Rotation of the Hydrochloride Prepared for Polarization.* In ordinary solvents, the solubility of an optical isomer is the same as that of its mirror image, but racemic compounds and racemic mixtures usually show greater or lesser solubility than their optically active components. To determine whether racemization could have produced

TABLE II  
*The Influence of Conditions of Heating with 25% H<sub>2</sub>SO<sub>4</sub> on the Optical Rotations of Arginine, Histidine and Lysine*

Hydrolyzate No.	Arginine Monohydrochloride				Histidine Methyl Ester Dihydrochloride				Lysine Dihydrochloride			
	Yield from flavinate	M.P. °C.*	[α] <sub>D</sub> °	Racemization	Yield from nitrate	M.P. °C.*	[α] <sub>D</sub> °	Racemization	Yield from picrate	M.P. °C.†	[α] <sub>D</sub> °	Racemization
	per cent		degrees	per cent**	per cent		degrees	per cent††	per cent		degrees	per cent†
R-24-1	50.0	212	21.00		57.7	202	9.80		77.0	199	15.55	
R-24-2						201						
A-120-2.5		212	20.95			204	9.70		85.0	200	15.45	
A-120-4	55.0	212	20.85		53.5	200	9.70			198	15.40	
A-120-18		212	20.90			202	9.75		70.0	198	15.50	
A-124-30	53.5	214	20.95		75.0	203	9.70					
A-140-1	56.0	212	20.95		58.0	204	9.73		65.0	198	15.50	
A-140-5	69.0	214	20.80		79.5	204	9.70		85.0	199	15.40	
A-140-14	48.5	212	19.90	5.24	65.0	201	7.50	23.5	90.0	197	15.45	
A-140-18	33.0	208	17.0	19.0	61.0	201	5.26	46.3	75.0	193	12.91	17.0
A-140-24	51.0	194	11.89	43.4	64.0	202	0.75	92.3	85.0	178†	8.70	44.1
A-160-24	49.0	192 (dec.)	0	100.0	60.0	202	0	100.0	30.0††		0.0	100.0

\* The melting point indicated in the table was the temperature (uncorrected) at which melting began. Most of the samples melted over a range of 2-3 degrees.

\*\* All active derivatives were dextrorotatory. The specific rotations of those prepared under the reflux were taken as standard for calculating the percentage converted to the *dl*-form. Only appreciable degrees of racemization are indicated.

† This derivative melted over a range of 6°C.

†† The unusually low yield here was due to an error in manipulation.

either appreciable concentration or appreciable loss of the *d*-isomer<sup>1</sup> in the involved process of isolation and conversion to the hydrochlorides used for polariscopic assay, the recoverability of additions of *d*- or *dl*-amino acids to casein hydrolyzates was tested.

A reflux hydrolyzate of casein prepared as before was divided into two equal portions. An aliquot equivalent to 4 g. of pure casein was removed from the first half for amino and total nitrogen analysis, and the balance was subdivided into two equal parts; to one of these was added *dl*-arginine, to the other none. The second half of the hydrolyzate was also subdivided into two equal parts, to one of which were added *d*-histidine and *dl*-lysine, to the other neither. The analytical data (Table III)

TABLE III

*Recovery of dl-Arginine, d-Histidine and dl-Lysine, after their Addition to a Casein Hydrolyzate, by Isolation as the Monoflavinate, Nitrilate and Picrate*

Aliquot No.	Casein Equivalent	Amino Acid Addition*	Wt. of Derivative Isolated	Amino Acid Equivalent	Calculated Recovery	
					g.	per cent
Ia	23	0.400 gm. of <i>dl</i> -arginine	3.517	1.255	0.411	102.8
Ib	23	none	2.364	0.844		
IIa	25	0.200 gm. of <i>d</i> -histidine	1.942	0.783	0.223	111.5
IIb	25	none	1.391	0.560		
IIa	25	0.800 gm. of <i>dl</i> -lysine	5.478	2.136	0.632	79.0
IIb	25	none	3.857	1.504		

\* The arginine was added as the monohydrochloride, the histidine as the monohydrochloride monohydrate and the lysine as the dihydrochloride.

indicated no loss of *dl*-arginine or *d*-histidine at the flavinate or nitrilate stage, but the recovery of added lysine as the picrate was incomplete. Calculations indicate that the limited solubility of the lysine phosphotungstate isolated initially was partly responsible.

To reduce the considerable loss entailed in preparing soluble hydrochlorides of suitable purity, the two arginine flavinate precipitates were combined, as were also the two precipitates of histidine nitrilate. The data recorded in Table IV suggest that the loss of the *d*-isomer of arginine or lysine did not greatly exceed the loss of the *l*-isomer, as did the loss of *d*-histidine. At least in regions showing approximately 20% racemization of arginine, 30% racemization of histidine and 30-40% racemization of lysine, the degree of racemization indicated in the

<sup>1</sup> The letter is used in this paper to indicate configuration.

TABLE IV

*Content of d-Amino Acid in Hydrochlorides of Arginine, Histidine and Lysine Isolated from a Casein Hydrolyzate to which d-Isomers of these Amino Acids had been Added*

Aliquot No.	Amino Acid Hydrochloride Isolated	Yield from Initial Derivative	$[\alpha]_D^{20}$	Content of d-isomer*	Content of d-isomer in initial ppt.
Ia+Ib	Arginine Monohydrochloride	<i>per cent of theory</i> 47.7	<i>degrees</i> 17.25	<i>per cent</i> 8.9	<i>per cent</i> 9.5
IIa+IIb	Histidine Monohydrochloride Monohydrate	50.5			
	Histidine Methyl Ester Dihydrochloride	20.2	8.0	8.7	14.9
IIa	Lysine Dihydrochloride	82.5	11.2	14.2	18.9 (14.8)**
IIb	Lysine Dihydrochloride	81.7	15.45	—	none

\* The letter as used here implies configuration.

\*\* The figure in parentheses is corrected for the 79% recovery of added *dl*-lysine at the picrate stage.

table for arginine and lysine is probably essentially correct, but that estimated for histidine is possibly too low.

The observation on histidine is subject to reservations because the yield of the methyl ester dihydrochloride in the test was unusually low. The proportionately greater loss of the added *d*- than of the original *l*-isomer is contrary to the slight concentration of the *d*-form noted by Conrad and Berg (10) in tests in which the total histidine was increased by the addition of 5% or less of this modification to a hydrolyzate or to rat carcasses subsequently hydrolyzed. The discrepancy may, of course, be due to differences in isolation procedures (10) or to the thrice as great initial concentration of the *d*-isomer used in the present instance. Duschinsky (22) has shown that at 20°C. and 40°C. *dl*-histidine monohydrochloride is a stable racemic compound which is transformed at 40–55°C. into a conglomerate of antipodes. By using relatively large amounts of histidine and varying temperature, concentration and solvent, he was able to isolate the *l*-, *dl*- and *d*-isomers in relatively pure form from a concentrated solution of the *l*- and *dl*-histidine monohydrochloride monohydrates. It seems quite probable, therefore, that the *d*-component of partially racemized histidine may be either lost or concentrated during the preparation of the intermediate monohydrochloride, depending upon its proportion originally in

the mixture and also upon the details of procedure followed for the isolation of this compound. Unfortunately, it has not been possible as yet to augment this phase of the study.

*Preparation of dl-Arginine Monohydrochloride.* On the basis of the procedures and the data presented earlier in this paper, the following is added as an outline of a method found convenient for preparing optically inactive arginine as the monohydrochloride:

170 g. of casein were autoclaved at 160°C. for 24 hours with 1 l. of 25% (by volume)  $H_2SO_4$ . The hydrolyzate was freed from sulfate ion with  $Ba(OH)_2$  and concentrated to 300 cc. The solution was cooled in the refrigerator over night and the amino acids which precipitated were filtered off. The filtrate was decolorized with charcoal, again filtered, and to the filtrate were added 24 g. of flavianic acid. After standing for 72 hours in the cold, the diflavanate (8.5 g.) was filtered off and converted to the monoflavanate. The latter was decomposed with  $HCl$  and the arginine converted to the monohydrochloride in the usual way. The yield of recrystallized arginine monohydrochloride was 1.728 g., equivalent to approximately 1.43 g. of arginine. The compound showed no optical rotation. It melted at 192°C. (uncorr.) and yielded 26.42% nitrogen (the calculated percentage is 26.6).

#### SUMMARY

Comparisons have been made of the susceptibility of arginine, histidine and lysine to destruction and racemization during the heating of casein with 25% sulfuric acid (by volume) longer than necessary to complete its hydrolysis in the autoclave at 120°, 140° and 160°C. The heating periods varied from 1 to 36 hours. Extent of destruction was estimated from the arginine isolable as the monoflavanate, the histidine as the nitrilate, and the lysine as the picrate from these hydrolyzates, as compared with the corresponding amounts of these derivatives separable from hydrolyzates prepared by the standard procedure of refluxing the casein with the acid for 24 hours. Degree of racemization was determined by comparing the specific rotations of the arginine monohydrochloride, the histidine methyl ester dihydrochloride and the lysine dihydrochloride into which the former derivatives were converted.

No appreciable destruction or racemization was observed before the minimum time for complete hydrolysis had been exceeded. The yields in 18 hours at 120°C. and in 5 hours at 140°C. were approximately maximal and about the same as from the standard reflux hydrolyzates, and the hydrochlorides prepared from these derivatives showed maximal specific rotations. Prolonging the heating decreased the yield of arginine most markedly, of histidine to a much lesser extent, and of

lysine apparently the least; the histidine was apparently the most readily racemized, but in 24 hours at 160°C. the racemization of all three seemed to be complete.

Known amounts of the *d*-histidine and *dl*-arginine which had been added to a test hydrolyzate to simulate racemization could be recovered completely as the monoflavinate and the nitrilate, but about a fifth of the added *dl*-lysine was lost. Calculation from the specific rotations of the arginine monohydrochloride and the lysine dihydrochloride subsequently prepared indicated that no appreciable change in the proportion of the *d*- to the *l*-isomer effected by the initial addition had occurred during the manipulations. An appreciable diminution in the proportion of *d*-histidine did occur in the course of preparing the methyl ester dihydrochloride, in contrast with previous, but not entirely analogous, observations. The results indicate that the procedures were probably adequate for comparative purposes.

A simple method of preparing *dl*-arginine monohydrochloride, based on the experiences gained in the study, is outlined.

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## Amino Acids in Turtle Egg

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### INTRODUCTION

In the course of comparative amino acid studies of the muscle proteins of different animal species (1), a relatively large supply of immature turtle eggs became available. It seemed advantageous to determine the amino acid composition of this material for several reasons. First, the turtle represents the reptilia, that class of animals next below aves in the taxonomic scale and the lowest vertebrate definitely committed to breathing air and depositing eggs on land. Second, the turtle is poikilothermic whereas the fowl is homothermic. Third, there is extensive information concerning the eggs of other animals—aves, amphibia and pisces—but there is little information on the eggs of reptilia.

### EXPERIMENTAL RESULTS

The eggs were taken from the bodies of a number of turtles (wood and painted) and were in an advanced stage of development. The material represents only the yolk, as the egg white and shell had not yet been deposited. The eggs were boiled prior to desiccation by the cryochem process, after which they were prepared and analyzed by previously published procedures (1). The results of lipid analyses of immature turtle eggs and turtle muscle have been reported elsewhere (2).

The proximate composition of turtle egg yolk and muscle are given in Table I. There is notably more moisture and less total solids in the muscle than in the egg. Although the greater concentration of solids in the egg yolk reflects higher amounts of protein, fat and ash, these are not proportionately greater in concentration, since the protein-fat ratio in the egg yolk is 1.5 and in the muscle is 6.9, *i.e.*, the egg yolk has not only a higher absolute amount of fatty material, but also a relatively greater proportion of fat to protein than the muscle. Approximately 53% of the total solids of the egg yolk is protein, whereas 70% of the muscle solids is protein.

The amounts of 10 amino acids in turtle egg yolk and muscle are given in Table II. The egg protein contained more arginine, phenylalanine, tyrosine and cystine but



TABLE I  
*Proximate Composition of Turtle Egg and Muscle*  
*Per cent of fresh weight*

	Moisture	Total Solids	Protein*	Fat†	Ash
Egg Yolk	50.2	49.8	26.2	17.6	2.5
Muscle	79.3	20.7	14.5	2.1	1.0

\* Nitrogen  $\times$  6.25.

† Summation of individual lipid constituents (2).

lower concentrations of histidine, lysine, tryptophan, threonine and methionine. The differences in arginine and the sulfur-containing amino acids appear to be most significant. The molecular proportions of the amino acids calculated in terms of tryptophan are also given in Table II. On the basis of two molecules of tryptophan there are greater numbers of molecules of each of the other amino acids, except

TABLE II  
*Amino Acids in Turtle Eggs and Muscle Protein*

	Protein*		Molecular Proportion (Ratio with respect to tryptophan)	
	Egg†	Muscle	Egg†	Muscle
	<i>per cent</i>	<i>per cent</i>		
Arginine	7.6	6.7	16.1	11.3
Histidine	2.2	2.3	5.2	4.4
Lysine	7.0	7.7	17.7	15.5
Phenylalanine	4.6	4.3	10.3	7.6
Tyrosine	4.9	4.6	10.0	7.5
Tryptophan	1.1	1.4	2.0	2.0
Serine	—	6.4	—	17.9
Threonine	4.4	4.9	13.7	12.1
Cystine§	2.5	0.6	3.8	0.7
Methionine	2.5†	4.1**	6.2	8.1

\* Fat-extracted dry weight corrected for ash and moisture, and nitrogen content calculated to 16%.

† Immature (yolk only).

‡ Calculated from total organic sulfur less cystine sulfur.

§ Sullivan method. Values of 1.8 and 1.28 were obtained for egg and muscle, respectively, by the Graff method (1).

\*\* Sullivan. A value of 3.0 was attained by the Beach-Teague method.

methionine, in the egg than in the muscle protein. Most significant, for every 2 molecules of tryptophan in egg yolk there were 4 of cystine, while in muscle there was one.

## DISCUSSION

The significance of these results resides in their contribution to comparative and developmental biochemistry. As has been pointed out (3): "It is only the birds which have been successful in producing an egg really well stocked with fat, though the reptiles clearly show an approximation to this achievement." The protein-fat ratio of 1.5 in the turtle egg yolk falls between the ratio of 1.0 or less (4) in the hen egg and 2.6 in the frog egg (3). This value agrees with the taxonomic relationship of these different classes of animals. The low protein-fat ratio of the turtle egg yolk, approximating that of the hen, is typical for terrestrial eggs, in contrast to the higher protein-fat ratio found in aquatic eggs (3). The major portion (77%) of the total lipid material in the turtle egg (2) is neutral fat or triglyceride. This compares with a value of 66% neutral fat in the total lipid material of the hen's egg (2) and is probably the major source of the energy used by the embryo in its development. In contrast, the developing frog and certain fish embryos use protein as a major source of energy (3).

The amino acid pattern of the turtle muscle is very similar to that of the muscle proteins of other species (1, 4). On the other hand, the amino acid composition of the immature turtle egg yolk appears to vary from that of the avian egg yolk (4). All of the amino acids that have been determined, with the exception of tryptophan, and possibly methionine, occur in higher concentration in the turtle egg yolk. The greatest difference appears to be in the arginine content. The arginine content of avian yolk protein, 6.8%, was comparable to that of the avian muscles, 7.0% (4), and also of turtle muscle, 6.7%. The turtle egg, however, contained 7.6% arginine.

## SUMMARY

Turtle egg yolk has been analyzed for 10 amino acids. All the amino acids which were determined, with the exception of tryptophan, and possibly methionine, occur in greater concentrations in the turtle egg yolk protein than they do in the hen yolk protein. The notable differences between the amino acid composition of turtle egg yolk and muscle protein are that the egg yolk contains more arginine and cystine.

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# Rapid Photocolorimetric Micro Procedure for Blood Sugar Using Copper Reduction with Perchloric Acid Deproteinized Filtrates

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## INTRODUCTION

Photoelectric determination of blood sugar based on copper reduction and subsequent formation of molybdenum blue is complicated by the instability of the color obtained when techniques such as those of Folin (1929) and Benedict (1, 2) are employed. Despite this difficulty, copper reduction procedures offer certain advantages over those based on other reactions. For this reason, a study has been made of the reactions involved and a modified procedure for blood glucose devised. In this, perchloric acid has been adopted as a protein precipitant (3). The acid filtrate obtained serves to adjust the pH of the alkaline copper tartrate to a range which reduces its sensitivity to glutathione and other non-sugar reducing substances. The molybdenum blue color, formed by the reaction of reduced copper with a modified phosphomolybdic acid reagent, is further stabilized by using *N* sulfuric acid as the diluent.

With the proposed modifications, the procedure attains the precision and stability of the methods based on reduction of ferricyanide and has, in addition, the advantage of being more rapid. The accuracy approaches that obtained when zinc or cadmium hydroxide is used as a protein precipitant without the limitations imposed by the latter reagents. The reaction can be employed as a macro or micro method merely by varying the ratio of perchloric acid to blood, and by the selection of an appropriate color filter.

## EXPERIMENTAL

*Reagents*

*3% Perchloric Acid.* 60 ml. of perchloric acid (60%) are diluted to 2 l. with distilled water.

*Alkaline Copper Tartrate (Folin (1929)).* Dissolve 140 g. anhydrous sodium carbonate in 800 ml. distilled water. Add 52 g. sodium tartrate and 44 g. sodium bicarbonate. Dilute to approximately 3 l. and shake until completely dissolved. Dissolve 20 g. pulverized copper sulfate ( $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ ) in a small amount of water. Add to the alkaline tartrate solution and dilute to 4 l. Mix and allow to stand 1-3 weeks.<sup>a</sup> Decant from the precipitate of cuprous oxide formed.

*Phosphomolybdic Acid Reagent.* Merck reagent grade phosphomolybdic acid ( $20 \text{MoO}_3 \cdot 2 \text{H}_3\text{PO}_4 \cdot 48 \text{H}_2\text{O}$ ) has been found satisfactory.

Dissolve 160 g. phosphomolybdic acid in 2 *N*  $\text{H}_2\text{SO}_4$  and dilute to 2 l. with 2 *N*  $\text{H}_2\text{SO}_4$ . Allow to stand 10 days at room temperature<sup>a</sup> and use the supernatant.

*Normal Sulfuric Acid.* 500 ml. conc. acid diluted to 18 l. with distilled water.

*Standards.* A 1% stock solution of glucose is made by dissolving 1 g. glucose in 100 ml. 3% perchloric acid. Standards ranging from 20 to 80  $\gamma$  of glucose/ml. are made by proper dilution of the stock with 3% perchloric acid.

*Micro Procedure*

One-tenth (0.1) ml.<sup>b</sup> of whole blood is pipetted accurately into 4.9 ml. of 3% perchloric acid. The mixture is shaken and filtered or centrifuged. To a Folin-Wu sugar tube are added 1 ml. of the clear filtrate, 1 ml. of distilled water and 2 ml. of copper reagent. A reagent blank is run simultaneously by substituting 1 ml. of 3% perchloric acid for the blood filtrate. The tubes are immersed in boiling water and heated for 8 minutes, then cooled for 2 minutes in water at room temperature. Two ml. of phosphomolybdic acid reagent are added and the reaction allowed to proceed for 2 minutes. The solutions are then diluted to 25 ml. with *N*  $\text{H}_2\text{SO}_4$ . After mixing, the solutions attain a maximum color within 5 minutes. Color densities are read on a photoelectric colorimeter, using a filter having maximal transmission at 660  $\mu$ , after setting the reagent blank to read 100% transmission.

<sup>a</sup> If solutions are not "aged," the molybdenum blue color intensity will slowly vary from day to day until a maximum is reached. If newly prepared reagents are used, the concentration factor must be determined daily until no further change occurs.

<sup>b</sup> Precision micro pipettes calibrated to contain 0.1 ml. at 20°C., like the Normax type supplied by Kimble Glass Co., are satisfactory.

## DISCUSSION

Employing the described procedure, the optical density of the final solutions proved to be a linear function of the glucose concentration. With the micro procedure, standards in the range 10–80  $\gamma$  of glucose fell along a straight line with a deviation of 1  $\gamma$  equivalent to  $\pm 5$  mg.-% (Fig. 1). The precision of the entire procedure operating under routine conditions, as indicated by check determinations of 3 different analysts, was  $\pm 9.4$  mg.-% in the range 0–150 mg.-% and  $\pm 12.6$  mg.-% from 151–480 mg.-%.

To assure exact reproduction of the standard curve with different lots of reagents, the alkaline copper-perchloric acid filtrate mixture should have a pH of 9.3. Variation in pH by  $\pm 0.1$  unit will introduce a shift in the standard curve. In practice the pH has been constant and has given little trouble. Occasional variations in the preparation of the copper reagent will make it necessary to restandardize the curve.

The use of perchloric acid as a protein precipitant offers the advantages of a stable, easily prepared and rapidly dispensed reagent. Control of hydrogen ion concentration of the filtrate to insure maximum reduction of the copper by the glucose is essential. Although the highly acid filtrate obtained by the use of perchloric acid could be neutralized, this involves a time-consuming step. The neutralization of excess acidity by the alkalinity of the copper reagent seemed an obvious solution. Investigation of the minimum acidity of perchloric acid necessary to produce complete precipitation of protein and yet permit maximum copper reduction showed that the copper reduction passed through a maximum when the pH was lowered. Moreover, a non-sugar reducing substance, like glutathione, passed through a minimum reduction phase in the same pH range. This occurred at a pH of 9.3. As was to be expected, the combination of perchloric acid and copper reagent gave sugar values close to those obtained by means of zinc or other metal hydroxides which remove non-sugar reducing materials.

In general, the values obtained by the perchloric acid method were 12–30 mg./100 ml. lower than those obtained by application of a tungstic acid precipitant. This corresponds to the range of saccharoid material in blood found by others, where reduction in tungstic acid filtrates was compared with that in filtrates prepared by use of zinc or other metal hydroxides.

The reduction of phosphomolybdic acid to molybdenum blue has been the subject of numerous studies (4, 5). In the analysis of phos-

phates by the reduction of the molybdenum complex, it has been shown that the intensity of color developed from phosphomolybdate depends on the acidity and temperature of the solution. If reduction takes place in a hot solution, the color reaches a maximum intensity with sulfuric acid at 0.4 *N*. Further increase diminishes the color until a plateau is reached between 0.9 and 1.4 *N* sulfuric acid. Similar conditions should

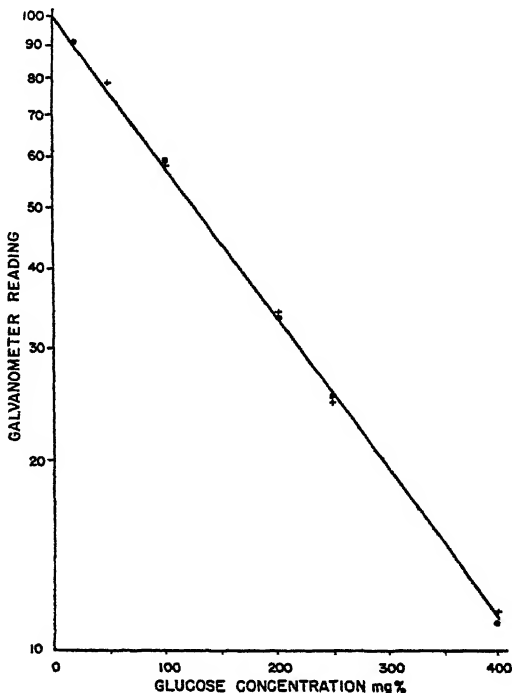


FIG. 1

The Relationship between Color Density (as Galvanometer Readings) of the Final Solution and Concentration of Glucose

hold for the molybdenum blue complex formed by reduction of phosphomolybdic acid with cuprous oxide. By adjusting the acidity of the reacting and diluting solutions with sulfuric acid as indicated, the color developed maintained a constant intensity after a considerable time interval. Whereas previously color comparisons made after 30 minutes were subject to error due to change in color intensity, the

technique described makes it possible to obtain constant readings over a period of 24 hours or more.

### SUMMARY

A micro or macro copper reduction procedure for the determination of blood glucose by photoelectric colorimetry is described. The use of perchloric acid as a protein precipitant offers a stable and easily dispensed reagent that yields protein-free filtrates having a pH, when mixed with the alkaline copper reagent, that renders them insensitive to saccharoid material. A modified phosphomolybdic acid reagent has been prepared from the commercially available chemical. With the glucose-reduced copper, the phosphomolybdic acid reagent yields a molybdenum blue color of such stability that constant readings can be obtained over a period of 24 hours.

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# A New Method for the Preparation of Basic Amino Acid Concentrates from Protein Hydrolyzates

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## INTRODUCTION

In agreement with the pioneer investigations of Osborne and Mendel (*cf.* 1), a survey (2) of the essential amino acid composition of the more common foods, indicated that the cereal proteins, especially those of wheat flour, had a considerably higher nutritive potential than is generally assumed. The data presented in Table I suggest that the addition of one amino acid alone, lysine, to white flour should be able to enhance the biological value of its protein two to three times before a secondary amino acid deficiency is encountered. This, in fact, has been demonstrated by feeding experiments in both our own and other laboratories (3, 4).

Therefore, a series of experiments were undertaken several years ago in an attempt to isolate lysine or a lysine-rich fraction from various animal and plant proteins such as casein, whey, blood meal, fish meal, soy bean meal, *etc.*, using the conventional procedures in the literature (5, 6). None of these methods gave entirely satisfactory results in our hands and the appearance on the market of synthetic ion exchange adsorbents seemed to offer promise of a new method for the separation of amino acids. A preliminary report on the preparation of a basic amino acid concentrate has appeared (7, 8).

## EXPERIMENTAL

### *Preparation of Ion Exchange Materials*

The synthetic cation exchangers used were the resin exchangers Amberlite IR-1, IR-100, XE-17, XE-47, and XE-12, Ionac C-284, Duolite C-3 and C-1, and the carbonaceous cation exchangers Zeo-karb and post-sulfonated Zeo-karb.

The anion exchangers, De-Acidite, Duolite A-3 and Amberlite IR-4, were also used in this study.

All cation exchange substances were converted to the hydrogen form by treatment with either 4% by weight of  $\text{H}_2\text{SO}_4$  or 5% of  $\text{HCl}$ , and the anion exchangers were converted to the free amine form with 2%  $\text{NaOH}$  or  $\text{Na}_2\text{CO}_3$  (cf. 9, 10).

It is our understanding that these synthetic cation resins are essentially sulfonated polymerized phenolic compounds with the exception of Amberlite XE-12 and XE-17, which are carboxylic resins. The permutit cation adsorbents are sulfonated coals and may contain either or both carboxylic and sulfonic acid groups.

#### *Column Adsorption of Amino Acids*

During the time that this investigation has been in progress, a great many columns were constructed varying in size from a narrow glass tube holding 5 g. of exchanger to a wooden barrel with a capacity of 220 lbs. In general, the results obtained using the 220-lb. column were the same as those obtained in laboratory-size glass columns.

### SEPARATION OF THE BASIC AMINO ACIDS BY THE USE OF CATION EXCHANGE ADSORBENTS

#### *A. Preparation of Hydrolyzate*

The initial experiments were carried out using Amberlites IR-1 and IR-100 and studying the adsorption of arginine hydrochloride, glycine and cystine, separately, in water or in dilute  $\text{HCl}$  solutions. The results did not indicate that the basic amino acids could be readily separated from the monoamino-monocarboxylic acids by the conventional technique (cf. 11).

Therefore, experiments using protein hydrolyzates were initiated. Fifty-gram quantities of the cation exchange substance in the 500 ml. dispensing burette were used for the majority of the experiments.

Ten parts of protein were hydrolyzed at atmospheric pressure with 25 parts of 9 or 18%  $\text{HCl}$  for 16–20 hours or with 22%  $\text{H}_2\text{SO}_4$  for 40–48 hours. It is necessary to achieve complete hydrolysis for the best results.

If 18%  $\text{HCl}$  is used as the hydrolyzing agent, the protein hydrolyzate is evaporated to dryness several times *in vacuo*. The acid residue is then dissolved in water and decolorized with activated carbon. If desired, a further quantity of mineral acid can be removed by passing the hydrolyzate over an anion exchange resin.

If  $\text{H}_2\text{SO}_4$  is used as the hydrolyzing agent, the excess acid can be removed with either barium or calcium hydroxides. Calcium sulfate has a significant solubility in the presence of certain amino acids and thus reduces the operating capacity of the cation exchange column. When  $\text{H}_2\text{SO}_4$  is used as the hydrolyzing agent, the  $\text{NH}_3$  can be removed from the hydrolyzates before adsorption of the basic amino acids, thus increasing the capacity of the column.

If  $\text{Ba}(\text{OH})_2$  is used to neutralize the acid, the hydrolyzate is adjusted to pH 3.6–4.1, care being taken to have an excess of sulfate ions at the end of the reaction. If

$\text{Ca}(\text{OH})_2$  is used, the pH is brought to 2.2 and as much  $\text{CaSO}_4$  is caused to precipitate as possible by working in concentrated solutions (15 to 20 mg. of N/ml.) and allowing the solution to stand over night, when possible. The acidity of pH 2.2 is not critical.

### B. Methods

Throughout this investigation the pH was determined with the Coleman glass electrode, nitrogen by the micro Kjeldahl method, while arginine was isolated as the flavianate, histidine as the nitraniolate and lysine as the picrate using a micro modification of the Kossel-Kutscher method (*cf.* 2).

### C. Adsorption of Basic Amino Acids

More important than the acidity of the amino acid influent is the concentration of the amino acids and the rate of flow. Within limits, the more dilute the influent amino acid solution and the more rapid the rate of flow, the better the separation of the basic amino acids.

The majority of experiments were carried out using solutions containing 2 mg. of nitrogen/ml., and were passed through a 50 g. or 150 ml. column at the rate of 50 ml./min. If the size of the column were doubled, the rate of flow was doubled. If the concentration were doubled, the rate of flow was halved. These relationships were found to hold over a concentration range of 1.7 to 17.6 mg. of N/ml. of influent. The reciprocal relationship between concentration of nitrogen and rate of flow is illustrated below:

Exp. No.	A Conc. of N mg./ml.	B Rate of Inflow ml./min.	A × B	Approx. Column capacity mg. of N
A75	1.72	150	258	1,700
A82	4.06	75	305	1,800
A83	5.76	50	288	1,700
A84	8.67	25	216	1,700
A85	17.6	13	229	1,800

The amino acid solution is allowed to flow downward over the column until the resin is saturated with basic amino acids. This is ascertained by showing that the composition of the effluent is the same, or almost the same, as that of the influent. In practice, two simple tests were used. The pH was determined using a glass electrode and the quantity of basic substances present were estimated by treating 2 ml. of the effluent with 2 ml. of 2% phosphotungstic acid in 5%  $\text{H}_2\text{SO}_4$ . The size and nature of the precipitates formed were compared with those in an equal aliquot of the influent at the same time and under the same

conditions. The results of a typical experiment are given below:

Influent: pH 4.3; N = 1.72 mg./ml.; P.W. = 15 +

No.	Effluent Vol. ml.	P.W. Test	pH	N in mg.
1	100	Neg.	2.0	13
2	200	Neg.	2.3	50
3	300	Neg.	2.8	113
4	400	3+	3.2	133
5	500	5+	3.4	146
6	600	10+	3.7	153
7	700	12+	4.0	156
8	800	12+	4.1	159
9	900	12+	4.1	161
10	1000	12+	4.2	164

It is obvious that considerable quantities of basic amino acids would be lost by this process if the effluent were discarded. However, that portion of the effluent and wash containing significant quantities of lysine, *etc.*, (Nos. 5 to 10), are passed over a fresh resin bed and new hydrolyzate then added to saturation. In this way, 80-90% of the lysine in a protein hydrolyzate can be recovered in the concentrate. The effluent, rich in nitrogen, though low in the basic amino acids, can be used for cattle feed, fertilizer, recovery of glutamic acid (*cf.* 2), *etc.*

#### D. Washing the Column

After the column is saturated, it is necessary to wash the exchanger very thoroughly with water, as 10-15% of the influent nitrogen will be removed by this process. It is preferable to use cation-free water for washing although, in localities where the cation content of the water is low, tap water can be used. It is best to wash with water equal in volume to 10-15 times the weight of the exchanger.

The rate of washing is usually 50 ml./min. for a 50 g. column. If dilute influents are used (N less than 6 ml./ml.), this rate is maintained over the entire run. If more concentrated influents are used (N greater than 6 mg./ml.), it is customary to wash initially at the same rate as the adsorption until a volume of water equal to that of the resin has passed through the column, when the more rapid rate is commenced. The column is backwashed for 10 or 15 minutes to prevent channeling and to remove suspended matter which has accumulated on the adsorbent. The rate of backwashing is adjusted to produce a 30-50% bed expansion (9). The value of thorough washing, if the pyridine elution method is not used (*cf.* below), is indicated by the following:

No.	Vol. ml.	N mg.	pH.	P.W.
1	400	1,960	2.4	5+
2	400	460	3.2	Neg.
3	400	180	3.4	Neg.
4	400	130	3.4	Neg.
5	400	80	3.4	Neg.
6	400	60	3.5	Neg.

*E. Elution of the Amino Acids.*

1. ACID ELUENTS. During the course of this investigation, concentrations of HCl ranging from 1-21% and of  $\text{H}_2\text{SO}_4$  from 0.75-10% were tried. The majority of the tests were carried out at room temperature ( $16^\circ$  to  $33^\circ\text{C}.$ ), although a few were done with hot acid ( $90^\circ$  to  $98^\circ\text{C}.$ ). Also, alcoholic HCl was used in one experiment without apparent gain. The excess acids are removed in the usual fashion.

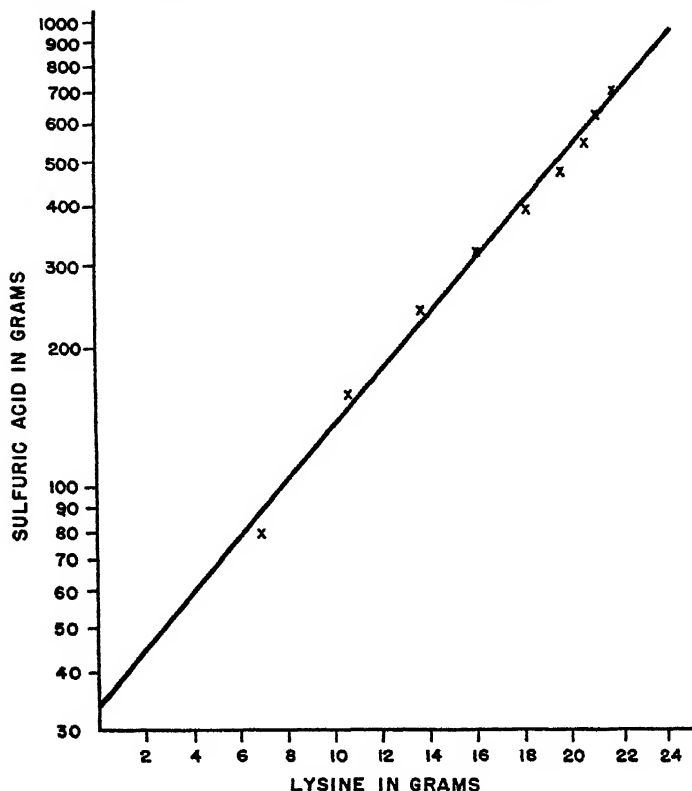


FIG. 1  
Elution of Lysine

Despite the complexity of the cation mixture yielded by acid hydrolysis of a protein, the results obtained on elution of a saturated cation exchanger appear similar to those found by Myers and Eastes (12) in the sodium-calcium exchange relationships on Amberlite IR-1. The data given in Fig. 1 indicate that lysine is removed from the cation exchanger according to the general parabolic curve represented by

$$y = a \log x + b$$

where  $y$  = yield of lysine and  $x$  = quantity of  $\text{H}_2\text{SO}_4$  required for elution.

*a. Recycling.* In an attempt to reduce the large quantities of sulfuric acid required to remove lysine, the effect of recycling was investigated. This was carried out in two ways. The first was simply to elute a column with 5 volumes of 4%  $\text{H}_2\text{SO}_4$  and return the eluate to the column until the solution has passed over the column 4 times. This procedure proved unsuccessful, as little added lysine was eluted after the first passage. The second method was to retain the second half of the elutriate and use this as the *first* portion for eluting another saturated column. This procedure was most useful and reduced the quantity of acid required by half. The increase in lysine on recycling the second half of the eluting acid is shown below:

Column: Amberlite IR-1 2,000 g.	Eluting Acid: 4% $\text{H}_2\text{SO}_4$
Protein: Blood Meal	Volume of Acid: 10 + 10 l.
Lysine content of first half of eluting acid	6,630 mg.
Lysine content of second half of eluting acid	2,470 mg.
Lysine content of first acid recycled on new column	9,740 mg.
Lysine content of second half of eluting acid (new)	2,540 mg.

It appears that the distribution of lysine between the eluting acid and the cation exchanger is in favor of the resin, so that, as the lysine adsorbed on the exchanger is decreased and that in the eluting acid is increased, a point is reached where no further quantities of lysine and other basic amino acids are removed. Thus, splitting the eluting acid into thirds or quarters and recycling them in the proper order resulted in worthwhile desorption only in the first and last portions, the last portion being fresh acid.

*b. Concentration of Acid.* It was observed that when the basic amino acids were eluted with  $\text{H}_2\text{SO}_4$  of strengths lower than 3% until no further quantities were removed, as shown by the phosphotungstic acid test, additional quantities of base could be eluted with 5% sulfuric acid.

Phosphotungstic acid precipitable N removed by 2.5%  $\text{H}_2\text{SO}_4$  to negative P.W.  
test: 2,106 mg.

Phosphotungstic acid precipitable N removed by 5%  $\text{H}_2\text{SO}_4$  to negative P.W.  
test: 720 mg.

Analysis of these two fractions for the three basic amino acids showed that the distribution of the amino acids in the 2.5 and 5% eluates varied markedly. These results and others are summarized in Table II. It is apparent that the removal of the basic amino acids from the cation exchanger is not in accord with their basicity. Thus, histidine, which is the weakest base, is removed approximately equally by both 2% and 5%  $\text{H}_2\text{SO}_4$ , while lysine, a stronger base than histidine, is definitely concentrated in the more dilute acid eluates. The reverse picture is seen with arginine, which is concentrated in the 5% sulfuric acid eluates. Ammonia, which was not present in

TABLE I

*The Relative Quantities of Essential Amino Acids Supplied by the Proteins of the Cereal Grains as Compared to a Milk-Meat Protein Standard*

Amino Acid	Supplied by 50 g. of Milk Proteins plus 50 g. of Meat Proteins	Relative Quantities* Furnished by 100 g. of Protein from			
		White Flour	Corn Grits	Oat Meal	White Rice
	<i>g.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Lysine	7.8	25	10	40	40
Isoleucine	7.4	50	70	75	70
Threonine	4.6	60	90	75	90
Valine	7.1	60	70	90	90
Arginine	6.0	65	50	100	120
Tryptophan	1.5	65	45	80	85
Cystine and Methionine	4.5	75	55	95	105
Leucine	9.5	80	250	85	95
Histidine	2.8	80	55	80	55
Phenylalanine	5.3	105	120	125	125

\* Values are rounded to nearest 5%.

TABLE II

*Influence of Eluting With Acids of Varying Strength and with Weak Bases on the Composition of the Eluates*

Protein	Eluting Acid H <sub>2</sub> SO <sub>4</sub>	Nitrogen Distribution			Comments
		Arginine N	Histidine N	Lysine N	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Casein	Original	8	3	9	Eluted with 5% acid only. 1.5% used to P.W. negative, then 5% acid
Casein	5%	27	12	31	
Casein	1.5%	22	17	41	
	5%	45	11	11	
Casein	2.5%	26	16	35	2.5% acid used to P.W. negative, then 5% acid
	5%	47	19	10	
Blood Meal	Original	7	8	9	Eluted with 4% acid only 2% acid used to P.W. negative, then 5% acid Pyridine Ammonia
Blood Meal	4%	23	25	35	
Blood Meal	2%	19	25	42	
Blood Meal	5%	30	24	18	
Blood Meal	Exchange	trace	43	trace	
Blood Meal	Exchange	43	trace	45	



any of the three hydrolyzates in Table II, is a slightly stronger base than arginine. Nevertheless, it is eluted by very dilute acid as shown below:

Ammonia removed by 0.75% $\text{H}_2\text{SO}_4$ to negative P.W. test:	240 mg.
Lysine removed by 0.75% $\text{H}_2\text{SO}_4$ to negative P.W. test:	1800 mg.
Ammonia removed by 4% $\text{H}_2\text{SO}_4$ to negative P.W. test:	0 mg.
Lysine removed by 4% $\text{H}_2\text{SO}_4$ to negative P.W. test:	1320 mg.

In fact, the removal of ammonia alone from cation exchange resins requires only 150–200% of the stoichiometric quantity of acid, being similar in this respect to the common inorganic cations (*cf.* 9, 10). In contrast, removal of the basic amino acids requires much larger amounts of eluting acid.

As a result of these experiments, it is believed that elution with either constant-boiling HCl or with 10–20 volumes (based on the weight of dry resin) of 4% sulfuric acid is the most feasible. The second half of the eluting acid is used as the first portion of the following run. The rate of elution does not appear critical; but, in practice, a rate of half the dry weight of the cation exchanger/min. is used.

2. CATION EXCHANGE. *a. Ammonia.* The observation that ammonia is much more readily removed from the cation exchange resin than the basic amino acids, led to the study of the effect of exchanging ammonia for amino acids. The adsorption of the bases and the washing of the column were carried out as usual but the amino acids were exchanged by passing an aqueous solution of ammonia (4%  $\text{NH}_3$ ) slowly over the column until the ammonia concentration of the effluent was approximately equal to that of the influent.

The ammonia was then either removed by elution with 4% sulfuric acid until the eluate gave a negative phosphotungstic acid test or a fresh amino acid hydrolyzate was passed over the column and the ammonia replaced by amino acids. Both procedures have their advantages and disadvantages. If ammonia—amino acid exchange is used, the amino acid effluent contains large quantities of ammonia which must be removed by distillation from lime ( $\text{H}_2\text{SO}_4$  hydrolyzates only) before the subsequent adsorption of lysine, *etc.* However, if the adsorbed ammonia is eluted with sulfuric acid, the column is in the hydrogen stage ready for the next cycle.

Successful exchange was effected using concentrations of  $\text{NH}_3$  over the range of 1–10%. During the adsorption of the  $\text{NH}_3$ , the column swelled appreciably (10–20%) and the advancing layer of ammonia down the column made itself evident by the heat produced. The ammonia-saturated column was washed with water and the effluent and washings were concentrated or the ammonia was removed by steam distillation. Due to the basicity of arginine and lysine, the resulting residue is free of ammonia. The residues were acidified to pH 4 with HCl or  $\text{H}_2\text{SO}_4$  and the solutions were decolorized by boiling with Darco S-51 or G-60.

The ammonia-saturated resin was then used for the next cycle or first eluted with 4% sulfuric acid as described above. No non-ammonia nitrogen was found in the

H<sub>2</sub>SO<sub>4</sub> eluates. The resin returned to its original volume both after H<sub>2</sub>SO<sub>4</sub> elution and amino acid exchange.

*The State of the Basic Amino Acids after Ammonia Exchange*

To have a rapid test of the efficacy of the resin exchange, the quantity of nitrogen precipitated from a 5 ml. aliquot of solution, containing 5–8 mg. of nitrogen, by 5 ml. of 40% phospho-24-tungstic acid in 5% H<sub>2</sub>SO<sub>4</sub>, in 3–4 hours at 0°C. was determined. The results of this test, on the *acid eluates*, correlated closely with the percentage of arginine, histidine and lysine nitrogen determined by the Kossel procedure. Thus, on 19 different adsorption experiments:

Base nitrogen in *per cent* of total nitrogen =  $71 \pm 4\%$  by P.W. test

Base nitrogen in *per cent* of total nitrogen =  $69 \pm 4\%$  by isolation method

When this same test was applied to the basic amino acid concentrates from blood hydrolyzates obtained by NH<sub>4</sub>OH exchange (Amberlite IR-1 or 100 but not Duolite C-1), no such correlation was found. Thus, on 17 different adsorption experiments:

Base N in *per cent* of total N =  $82 \pm 3\%$  by P.W. test.

Base N in *per cent* of total N =  $45 \pm 4\%$  by isolation method.

These values were significantly different, and showed either that more non-basic nitrogen than usual was being precipitated by the phosphotungstic acid procedure or that the Kossel method as usually applied did not isolate all the basic amino acids from the ammonia eluate. Experiments showed that both these explanations were correct.

The distribution of basic amino acid nitrogen in a 4% sulfuric acid eluate of a blood hydrolyzate usually ran:

Arginine N = 18–23% of total N

Histidine N = 21–25% of total N

Lysine N = 25–35% of total N

In contrast the nitrogen distribution in a typical ammonia eluate was:

Arginine N = 10–12% of total N

Histidine N = 18–24% of total N

Lysine N = 13–15% of total N

The yield of histidine nitranilate in these experiments was approximately that expected, but the yields of arginine flavianate and lysine picrate were decidedly lower. In several instances, concentration of the filtrates from the arginine flavianate and lysine picrate precipitates yielded orange and red oils, respectively.

It was first thought that these amino acids were present as ammonium salts or amides. However, no extra ammonia was yielded either by direct distillation from alkali or after hydrolysis with 2 *N* or 8 *N* sulfuric acid.

In fact, Kossel analysis of a sample of ammonia eluate hydrolyzed for 15.5 hours with either 2 *N* HCl or H<sub>2</sub>SO<sub>4</sub> gave essentially the same amino acid picture as before hydrolysis. However, if the eluates were hydrolyzed with 8 *N* H<sub>2</sub>SO<sub>4</sub> over night, no difficulty was encountered in isolating the three basic amino acids. Thus, ammonia experiment A62:

	Arginine N	Histidine N	Lysine N
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Before Hydrolysis	10	18	13
After Hydrolysis	18	18	31

In other experiments, the oils obtained from the arginine flavianate and lysine picrate precipitates could not be crystallized. When these oils were dissolved in water, the dye removed and the solutions boiled with H<sub>2</sub>SO<sub>4</sub> (8 *N*) over night, arginine flavianate and lysine picrate crystals were obtained immediately under the usual conditions. In spite of many experiments, an explanation of this phenomenon has not been found. There is no good evidence of peptide formation.

*b. Pyridine and Ammonia.* Although the primary objective of these experiments was to prepare a lysine concentrate by the simplest procedure, the possibility of direct chromatography and of selective elution of the basic amino acids was always kept in mind. Some success along this line was achieved by consecutive elution with acids of different concentrations (*cf.* 1b. above), while very dilute ammonia was able to elute histidine with little coelution of arginine and lysine. However, elution with a weaker base, such as pyridine (*cf.* 14 for literature), proved more useful.

Aqueous pyridine ranging in concentration from 5 to 50% is selectively exchanged for histidine and the monoamino acids. The pyridine eluate is concentrated to recover the pyridine. The arginine, lysine and pyridine which remain on the resin are then exchanged for am-

monia by the process given above. The following experiment is typical:

Histidine and monoamino acids removed by 8%  $C_5H_5N$  to negative Pauly test:

Total N eluted = 484 mg.; while 776 mg. of histidine were isolated from this fraction as the 3, 5-dichlorosulfonate.

Arginine and lysine removed by  $C_5H_5N$  to negative Pauly test:

None found by Kossel and Sakaguchi analysis.

Histidine removed by 4%  $NH_3$  following pyridine elution to negative Sakaguchi test:

None by Kossel analysis, trace by Pauly test.

Arginine and lysine removed by ammonia accounted for 43% and 45%, respectively of the total N of this fraction.

#### *F. Cation Exchange Materials*

All the exchangers used in this study were devised primarily for the removal of inorganic cations from very dilute aqueous solutions and, although they are all effective for that purpose, they are not of equal value for the concentration of basic amino acids from protein hydrolyzates.

The difficulty of removing the adsorbed basic amino acids from the sulfonic acid type resins (Amberlite IR-1, IR-100, XE-17, Ionac C-284, Duolite C-1 and C-3), led us to investigate resins with free carboxyl groups, Amberlite XE-12 and XE-47. We were surprised to find that Amberlite XE-12 failed to concentrate any appreciable quantities of basic amino acids either from blood hydrolyzates of pH 2.2-2.6 or of pH 6.0 under our experimental conditions; XE-47 failed to concentrate at pH 2.2, but worked satisfactorily on very dilute influents of approximately pH 6.0.

Likewise, no concentration of arginine, histidine and lysine was achieved with the sulfonated coals, Zeo-karb and post-sulfonated Zeo-karb, under the experimental conditions, although these carbonaceous exchanges did adsorb both mono- and polyamino acids.

### RESULTS

Basic amino acid concentrates have been prepared from blood meal, casein, fibrin, whey residue (lactalbumin) and alkali-soluble soy proteins. In each case, the original hydrolyzate and the final base concentrate were analyzed by the small-scale Kossel procedure (*cf.*

2). Some results are given in Table III. Table IV gives a more detailed analysis of the amino acids in the original blood meal hydrolyzate and in a 4%  $\text{H}_2\text{SO}_4$  eluate prepared from it. The analytical methods used have been described (cf. 2).

TABLE III  
*Basic Amino Acid Nitrogen Distribution in Proteins and in the Cation Exchange Resin Eluates*

Source	Protein Nitrogen in per cent of total Nitrogen				Cation Resin Eluate Nitrogen in per cent of total Nitrogen			
	Arg. N	Hist. N	Lysine N	Total Base N	Arg. N	Hist. N	Lysine N	Total Base N
Casein	8	3	9	20	27	12	31	70
Fibrin	15	4	9	28	30	10	22	62
Whey	9	3	9	21	29	10	29	68
Soy	12	4	7	23	32	11	20	63
Blood	7	8	9	24	16	22	29	67
Blood	7	8	9	24	23	25	35	83
Blood	7	8	9	24	>1	>1	>1	>2 Effluent from above experiment

TABLE IV  
*The Effect of Chromatographing a Blood Protein Hydrolyzate on Nitrogen Distribution*

Amino Acid	Amino Acid N in per cent of total Nitrogen	
	Original Blood	Cation Exchange Eluate
Arginine	7.4	22.6
Histidine	8.3	25.3
Lysine	8.6	35.3
Tyrosine	1.8	0.5
Phenylalanine	3.3	2.0
Cystine	1.3	0.0
Methionine	0.9	0.4
Threonine	4.8	0.4
Leucine	11.6	3.1
Valine	4.5	1.6

A. *Isolation of Lysine Picrate* (cf. 6). Five hundred g. of a basic amino acid concentrate prepared as a paste ( $N = 9.0\%$ ;  $H_2O = 33\%$ ), were dissolved in 1 l. of tap water and heated to  $85-90^\circ C$ . Then 135 g. of picric acid, recovered from the decomposition of lysine picrate, were added and the heating continued until all the picric acid had dissolved. The solution was cooled with stirring. Crystals of lysine picrate appeared when the temperature dropped to  $60$  or  $65^\circ C$ . When the solution reached  $40^\circ C$ , the yellow needles formed a solid mass. After cooling to room temperature, the precipitate was filtered off, washed with cold water and dried. Arginine and histidine have been prepared from these filtrates. Yield 200 g.; explosion point  $252^\circ C$ . (uncorr.). Recrystallized: yield 184 g.; explosion point  $256^\circ C$ . (uncorr.).

Lysine picrate can be converted into the dihydrochloride in the following manner: 260 g. of lysine picrate (E.P.  $252^\circ C$ . uncorr.) were poured into a boiling mixture of 520 ml. of water and 43 ml. of conc. HCl. The lysine picrate dissolved in a few minutes. Then 118 ml. of conc. HCl were added to the boiling solution. Light yellow picric acid precipitated immediately. The solution was cooled to  $4^\circ C$ . and the precipitate was removed by filtration. After thorough washing with cold water, the picric acid was dried in the air. Yield 157 g.; theory 158.6 g.

The pale yellow filtrate was concentrated to dryness *in vacuo* to remove excess HCl. The residue was taken up in water and the solution decolorized either by 6 g. of activated carbon or by extraction with toluene. The colorless lysine solution was again concentrated to dryness and taken up in 400 ml. of hot  $95\%$  ethanol. Lysine dihydrochloride was precipitated from the cooled alcoholic solution by pouring into several volumes of cold ether (3). Yield 125 g.; theory 151 g.; M.P.  $191^\circ C$ . (uncorr.).

B. *Separation of Histidine, Arginine and Lysine*. It is obvious that the concentrates prepared by ion exchange should lend themselves to the isolation of histidine, arginine and lysine by conventional methods. Histidine is readily precipitated by 3,5-dichlorosulfonic acid (cf. 14), arginine by flavianic acid (cf. 2) and lysine by picric acid (2, 6). Then anion and cation exchange resins can be used to separate the precipitating acid from the amino acid.

For example, if a warm aqueous solution of lysine picrate is passed through an anion exchange column in the form  $RNH_2 \cdot H_2CO_3$  or  $RNH_2 \cdot HOOCCH_3$ , picric acid is exchanged for  $CO_2$  or  $CH_3COOH$ . The end-point of the reaction is easily ascertained by the color of the effluent. On the other hand, if one wishes to employ a cation exchange resin, the following experiment is illustrative: A solution of lysine flavianate, obtained from the removal of arginine flavianate, was passed over a cation exchange column in the hydrogen cycle until a positive phosphotungstic acid test for lysine was obtained in the effluent. The column was then drained, thoroughly washed with water and the lysine eluted with either HCl or  $NH_4OH$  as described above. Flavianic acid was recovered from the filtrate by concentration. Flavianic acid can be prepared from naphthol yellow S (sodium flavianate) by the same general method.

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## SUMMARY

1. Various cation exchange resins have been successfully employed to prepare concentrates of arginine, histidine and lysine from commercially available protein sources.

2. The cation-free effluents are suitable for the preparation of the dicarboxylic amino acids by anion adsorption.

3. The adsorbed dibasic amino acids can be simultaneously or, to a certain extent, selectively removed from the resins by elution with acids of varying concentration or by exchange with different weak bases.

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# The Estimation of Glycogen in Whole Blood and White Blood Cells\*

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## INTRODUCTION

In the course of studies on the glycogen content of whole blood and white blood cells in glycogen storage disease, leukemia and polycythemia, an improved technique for determining blood glycogen and its quantitative evaluation in isolated blood cells has been developed. The experimental data are presented only so far as the normal standards are concerned.<sup>1</sup> The total of the reducing substances derived from acid hydrolysis following alcohol precipitation was determined by a procedure which is essentially a micromodification of the Pfüger (1) method. For identification of the glycogen the acid hydrolyzates were subjected to yeast fermentation in order to determine non-sugar reducing substances. This method was then applied to whole blood, blood plasma, isolated white blood cells, blood platelets and red blood cells of normal as well as pathological individuals.

Although Pfüger included in his studies the determination in whole blood, few studies on blood were carried out in subsequent years. The literature on this subject prior to Pfüger's studies is reviewed in his monograph. The more recent literature on this subject is given in the bibliography of this paper (Nos. 2 through 8). Most of the investigators used acids as hydrolyzing agents, assuming that the total reduction in blood following alcohol precipitation and subsequent acid hydrolysis originated from glycogen. Gabbe (7) and Kuzin and Makaeva (8) applied diastatic enzymes instead of acid hydrolysis.

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<sup>1</sup> The results derived from pathological material will be published elsewhere.



DETERMINATION OF GLYCOGEN IN BLOOD AND  
BLOOD CONSTITUENTS

The following method was finally adopted for the determination of glycogen in blood and its constituents. The procedure is carried out in a specially made Pyrex glass test tube of 50 cc. capacity with ground glass stopper. As an anticoagulant 2 mg. of dry potassium oxalate per cc. of blood are used. When dealing with larger amounts of blood, as in the study of isolated white blood cells, we used 0.2 cc. of a mixture of 3% ammonium oxalate and 2% potassium oxalate per 5 cc. of blood, which is allowed to dry on the bottom of the container in which the blood is collected. Each determination is carried out on 1 cc. of whole blood. The blood is hemolyzed by pipetting it into 2 cc. of distilled water. After the addition of 3 cc. of 60% KOH, the mixture is heated for approximately thirty minutes in a boiling water bath. After cooling to room temperature and adding 6 cc. of water, the glycogen is precipitated with 18 cc. of 95% ethanol. It is important for the yield to allow the precipitate to settle over night. On the following day, the suspension is centrifuged for 25 minutes at a speed of approximately 2000 r.p.m. After 2 washings with 60% alcohol the last supernatant is always colorless. The precipitate is then hydrolyzed with 4 cc. of 2.2% HCl for 3 hours. The test tubes are covered with well fitting glass bulbs during the heating with KOH as well as during the hydrolysis with HCl. After neutralization with 2 N NaOH, using one drop of phenol red as indicator, the hydrolyzate is made up to 10 cc. with distilled water in a volumetric flask, centrifuged and the sugar determined in an aliquot (9 to 9.5 cc.) according to the Somogyi (9) method.<sup>2</sup> The results are recorded in mg. glucose/100 cc. of blood.

In another 1 cc. sample of whole blood the same procedure is carried out, but the hydrolyzate is fermented for one hour in the incubator with 0.5 cc. of a 10% aqueous suspension of yeast<sup>3</sup> before the sugar determination. In the analysis of isolated blood cells the total hydrolyzate is transferred to a 10 cc. volumetric flask and subdivided into two equal portions, one for direct determination and the other for determination after yeast fermentation. The difference between the two samples represents the glycogen content of whole blood or of the isolated blood cells examined.

The fermentation is carried out at pH 4-6. For fresh yeast the optimum of acidity is broad. It is convenient to use a 1% solution of  $\text{KH}_2\text{PO}_4$  as buffer thus increasing the fermenting power of living yeast. The hydrolyzate is neutralized with 2 N NaOH until phenol red turns red; the final adjustment with the 1% solution of  $\text{KH}_2\text{PO}_4$  being made to a yellow pink tinge. Under these conditions the pH is 4.9, according to electrometric determinations. We found in control experiments with glucose solution

<sup>2</sup> Most of the previous investigators (van Creveld (2), Golandas (4), Bridge and Holt (5) applied the Hagedorn-Jensen (10) method. Ellis & Payne (3) used the Folin (11) method. However, owing to its higher specificity the Somogyi method was found to be preferable for work on blood (*cf.* also Good, Kramer and Somogyi (12)).

<sup>3</sup> The suspension is made from ordinary Fleischmann's bakery yeast, which is washed 10 to 12 times with distilled water to rid it of reducing substances. We convinced ourselves in each experiment that the reduction of the yeast was negligible, if the Somogyi method was used.

that the fermentation in the neutralized hydrolyzate is less complete than in water. The discrepancy is constant for each individual sample of yeast, but varies from 4-12% for different batches of yeast. For this reason, the influence of the salt concentration of the hydrolyzate as a medium for the fermentation is determined in each individual experiment.

All determinations of glycogen in whole blood and isolated blood cells should be carried out on samples collected from fasting individuals.

## EXPERIMENTAL

### *1. Glycogen Determinations on Whole Blood*

As ample experimental data of previous investigators using the Hagedorn-Jensen method for the sugar determination are available, only a few experiments were carried out with this method, including the modifications described in the previous section. Thirteen normal individuals gave "blood glycogen" values averaging 11.7 mg.-% with variations from 7.9 to 20.9. This is in agreement with recent studies of Bridge and Holt (6). Their average derived from 10 normal individuals was 13.5 mg.-% with variations from 5 to 22. The value of 11.7 mg.-% cannot be considered as true glycogen, since it includes a certain amount of unfermentable reducing substances. This could be demonstrated in all determinations. Only 68% of the total reducing substances were proved to be fermentable with yeast. Thus, the true glycogen value in normal whole blood is reduced to 7.95 mg.-% if the sugar is determined according to Hagedorn-Jensen.

The glycogen values obtained by the Somogyi method are lower than the Hagedorn-Jensen values. The average of 42 determinations on 28 normal individuals amounted only to 5.5 mg.-% with variations from 1.2 to 16.2. In 32 of these determinations the range was between 1 and 7 mg.-%. Yeast fermentation did not reveal any non-sugar reducing substances. This average of 5.5 mg.-% is 31% lower than the corrected Hagedorn-Jensen value, since the copper reagent is more specific than the ferricyanide.

### *2. Glycogen Determinations on Isolated White Blood Cells*

The white blood cells are separated in a Cushman (13) tube after the total red cell volume is determined in a hematocrit tube. For normal blood with a white blood cell count of 5-10,000/mm.<sup>3</sup> the optimal amount of blood is 12 cc. After centrifuging for about half an

hour<sup>4</sup> at a speed of at least 3000 r.p.m., a sufficient amount of white blood cells can be secured easily. It is sufficiently exact to remove most of the creamy layer of the blood platelets since it is immaterial if there is a small remainder of blood platelets on top of the white blood cells. The reducing value of platelets is negligible, if the Somogyi method is applied.

White blood cells contaminated with platelets to a relatively small extent can be collected if the white blood cell layer is placed just at the upper part of the stem of the Cushman tube where it widens into the upper bulb. This is possible by selecting the proper Cushman tube and by using the appropriate amount of blood. Under these circumstances the blood platelets are arranged as a concentric ring on top of the white blood cell layer, which can then be easily withdrawn from its center by a capillary tube.

Since further separation of the white blood cells from the remainder of the plasma and the blood platelets is unnecessary, the material is immediately transferred into the weighed Pyrex test tube and suspended in 3 cc. of distilled water, followed by the regular procedure of glycogen determination. The weight of the average wet white cell layer derived from 12 cc. of blood is about 100 to 150 mg. In leukemia cases with high white blood cell counts as much as 500 to 800 mg. can be obtained.

It will be shown later that isolated white blood cells are much more susceptible to the action of enzymes present in blood than whole blood specimens. The addition of 0.03% NaF prevents the breakdown of glycogen to a great extent. It is, nevertheless, important to complete the entire procedure from collecting the blood until contact of the white blood cells with KOH within 1-1.5 hours.

For the quantitative evaluation of the result, the white blood cells in the layer are counted. The first subdivision of the blood pipette is calibrated with mercury. Both the weight of the total white blood cell layer and the weight of its amount in the blood pipette are determined.<sup>5</sup> The amount of glycogen per million wet white blood cells is then calculated according to the following formula:

$$\text{Glycogen per million white blood cells} = \frac{A \times B}{C \times D \times E} \gamma$$

A.....Glycogen found in the white blood cell layer ( $\gamma$ ).

B.....Weight of the white blood cell layer used for the cell count ( $\gamma$ ).

C.....Volume of the first subdivision of the blood pipette (mm.<sup>3</sup>).

D.....Number of white blood cells in the layer (millions per mm.<sup>3</sup>).

E.....Weight of the white blood cell layer ( $\gamma$ ).

The glycogen content of isolated white blood cells was studied on 22 normal individuals. The average content was 2.54  $\gamma$  per million

<sup>4</sup> Although desirable from the viewpoint of the quantitative yield of white blood cells and their better separation from the red blood cells, a longer period of centrifuging is not advisable because of the greater risk of an enzymatic destruction of glycogen.

<sup>5</sup> This technique seems to be rather complicated, but has the advantage of saving several time-consuming manipulations of the leucocytes.

cells, with variations from 0.96 to 3.80  $\gamma$ . In nine of the 22 specimens the glycogen content was between 2 and 3  $\gamma$ , in 6 specimens below 2, and in 7 above 3  $\gamma$ .<sup>6</sup>

### 3. Reducing Substances in Blood Platelets

In a short time it is not possible to secure white blood cells which are not contaminated by a small remainder of blood platelets. Purifying white blood cells of blood platelets is a lengthy procedure during which glycogen is lost because of the strong enzymatic activity in the white blood cell layer. Thus, it was necessary to examine purified blood platelets for reducing substances.

Wet blood platelets not contaminated by white blood cells may be collected by a procedure of fractional centrifugation.<sup>7</sup> The combined grayish-red and creamy layers in the Cushman tube are suspended in an ice cold 0.8% NaCl solution containing 0.6% sodium citrate (see Chargaff *et al.* (14)). This is best done in narrow cylindrical tubes such as hematocrit tubes of 1 cc. capacity. After standing for several hours in the refrigerator the suspension will show a grayish-red bottom layer of white blood cells, which are still contaminated with a few red blood cells and blood platelets, and a cloudy supernatant containing only blood platelets. After centrifuging at low speed for 10 minutes the suspension of blood platelets is withdrawn. It is practically free of white blood cells, containing not more than 1 white blood cell per 3000 blood platelets. This should be verified microscopically. The same procedure is repeated as long as blood platelets can be recovered from the grayish-red layer.

For determining the reducing substances of the blood platelets the combined turbid suspensions are centrifuged at high speed on the angle centrifuge, the blood platelets transferred into the Pyrex test tube, and suspended in 3 cc. of distilled water; in this suspension the glycogen is determined by the regular procedure. The number of blood platelets per mm.<sup>3</sup> must be counted and the calculation of the glycogen content is the same as in the case of the white blood cells.

Some information on the nature of the nonfermentable reducing substances in the hydrolyzates of platelets were obtained on larger amounts of dried and purified platelets prepared from horse blood according to the method of Chargaff *et al.* (14). Two specimens of dried blood platelets were collected for analysis. The weight of the first

<sup>6</sup> As far as the exactness of the method is concerned, duplicates were analyzed with satisfactory results in some instances where enough material was available. In some experiments white cell layers of different density and with admixture of a few red blood cells were studied. The differences did not exceed 0.08  $\gamma$  per million.

<sup>7</sup> Only if present in excessive amounts and if separated from the white blood cells by a long period of centrifuging at high speed, fairly well purified blood platelets can be withdrawn from the Cushman tube without any subsequent procedure of isolation.

amounted to 14.7 mg.; it was used for the determination of glycogen. The weight of the second specimen was 9 mg.; this was used for determining the total nucleic acid phosphorus and the pentose nucleic acid phosphorus.

The acid hydrolyzate of the 14.7 mg. specimen was subdivided into two equal portions. In one portion 252  $\gamma$  total reducing substances were found, using the Hagedorn-Jensen method. After fermentation the same amount was found again. Hence, there was no measurable amount of glycogen in dried platelets.

In the 9 mg. specimen 0.0285 mg. of total nucleic acid phosphorus were determined in one portion; in another portion the pentose nucleic acid phosphorus was determined and found to be 0.0282 mg.<sup>8</sup> Thus, the platelet nucleic acids consist entirely of ribonucleic acid. The order of

TABLE I  
*Reducing Substances in Wet Human Blood Platelets*

No. of Experiment	Weight of material	Platelets millions per mm. <sup>3</sup>	Reducing substances without fermentation		Method of sugar determination	Remarks
			Total	Per million platelets		
135	mg. 23.1	5.00	$\gamma$ 31.8	$\gamma$ 0.23	Somogyi	Not enough material for fermentation.
137	66.1	1.20	12.0	0.20	Somogyi	Not enough material for fermentation.
143	56.3	2.40	28.6	0.20	Somogyi	Not enough material for fermentation.
145	53.0	1.92	12.6	0.18	Somogyi	Reducing substances unfermentable. 47.1 mg. material yielded 13.5 $\gamma$ reducing substances after fermentation.
148	190.6	0.80	0.0	—	Somogyi	Material collected from 4 normal individuals.

<sup>8</sup> This analysis was carried out by Dr. G. Schmidt (15).

TABLE I (continued)

No. of Experiment	Weight of material	Platelets millions per mm. <sup>3</sup>	Reducing substances without fermentation		Method of sugar determination	Remarks
			Total	Per million platelets		
151	mg. 177.4	1.20	γ 50.0	γ 0.20	Hagedorn-Jensen	Reducing substances unfermentable. 80.5 mg. material yielded 20.4 γ reducing substances after fermentation.
152	282.0 184.0	0.75	0.0 0.0	— —	Hagedorn-Jensen	Material collected from 4 normal individuals.
153	140.6	0.68	0.0	—	Hagedorn-Jensen	
159	53.5	3.30	43.4	0.21	Hagedorn-Jensen	Reducing substances unfermentable. 32.2 mg. material yielded 28.7 γ reducing substances after fermentation.
160	67.3	2.70	50.0	0.29	Hagedorn-Jensen	Reducing substances unfermentable. 40.3 mg. material yielded 28.9 γ reducing substances after fermentation.
165	14.0	6.85	23.0	0.20	Hagedorn-Jensen	Not enough material for fermentation.

magnitude of the ribonucleic acid content of<sup>1</sup> platelets explains at least a large part of the reduction obtained after acid hydrolysis. Further evidence for the presence of considerable amounts of pentose compounds in platelets is the strongly positive Bial's test obtained on the acid hydrolyzate of platelets.<sup>9</sup>

<sup>9</sup> It was experimentally shown that nucleic acid which has undergone the same chemical treatment as glycogen (heating with potassium hydroxide, alcohol precipitation, washing with alcohol and acid-hydrolysis) gave a strongly positive Bial's reaction.

Several determinations on human blood samples showed similar results. All determinations were carried out on wet platelets. The results are shown in Table I. They are consistent with those obtained from dried platelets of horse blood. The reducing substances in the hydrolyzates of platelets were proved to be unfermentable (Exps. 145, 151, 159 and 160). In our technique of collecting white blood cells the creamy layer of blood platelets on top of the leucocytes is removed. A small remainder can be neglected in glycogen determinations on white blood cells.

### DISCUSSION

The values of glycogen in whole blood obtained by the earlier procedures are usually higher than those reported in this investigation. This discrepancy is explained by the higher specificity of the Somogyi method used in our analyses as compared with other reductometric methods, especially that of Hagedorn and Jensen. In addition, the error caused by the presence of reducing contaminations was eliminated in our analyses by fermentation controls.

It has been shown in this study that the leucocytes carry at least a considerable share of the total blood glycogen. Differences in blood glycogen are primarily the result of differences in the number of white blood cells per mm.<sup>3</sup> and their capacity to store glycogen. According to Hoppe-Seyler (16) glycogen is never absent as a constituent of white blood cells, as long as the cells are in living and contractile conditions showing ameboid movements; in the dead corpuscles of pus no glycogen was discovered. However, other investigators (Cramer (17) and Huppert (18)) were able to demonstrate glycogen in pus. In the present studies it was likewise detectable in pus, but its stability was different from that in white blood cells isolated from blood. The detectability of glycogen in pus depends on the conditions and the enzymatic activity of the leucocytes in this material.

In computing the results from the layer of the white blood cells, it was assumed that this layer is homogeneous and the glycogen equally distributed throughout all its elements. However, a white blood cell layer recovered from normal blood represents a mixture of biologically different cell elements, the two main types being the granulocytes and the lymphocytes. From experimentation on leukemic blood with one uniform cell type, results yielded data as to the glycogen content of the

different types of white blood cells. Neither blast forms nor lymphocytes contain any measurable amounts of glycogen. It is the granulated cell form, from the myelocytic stage of development on, which is the main carrier of glycogen in blood and therefore determines the glycogen content of whole blood. An average of 2.54  $\gamma$  glycogen per million white blood cells was found in the total mixed layer of normal blood specimens. Assuming an average of 60% granulated leucocytes this value has to be corrected to 4.23  $\gamma$  glycogen per million granulated cells.

The average glycogen content determined in whole blood exceeded that computed from the glycogen content per million white blood cells and the number of these cells per 100 cc. of blood in almost all instances. Special studies were carried out to determine whether or not this deficit can be explained by the presence of glycogen in plasma or red blood cells or both. However, in neither could glycogen be detected in measurable amounts.<sup>10</sup> Hence, the conclusion is justified that blood glycogen is only a constituent of the granulated white blood cells.

The glycogen content of the granulated mature leucocytes varies considerably in cells obtained from different specimens of blood. This is partially caused by the rapid enzymatic destruction of glycogen in the leucocytes, especially after their separation from the other blood constituents. Since the isolation of leucocytes from blood requires considerable time, the losses of glycogen due to enzymatic cleavage cannot be avoided. Chart I shows the results of some typical experiments on the rate of the enzymatic disappearance of glycogen in whole blood, leucocytes and pus under various conditions. The breakdown of glycogen in isolated white blood cells has an entirely different rate from that in whole blood and pus; 93% of white blood cell glycogen was destroyed after 24 hours at refrigerator temperature (+ 4°C.). It is the same rate of disappearance known to occur in other organs with active glycogen metabolism, such as the liver. In pus the rate of disappearance more nearly resembles that in whole blood.

Since, under comparable conditions, glycogen disappears in isolated leucocytes at a much faster rate than in whole blood, it is obvious that the amount of glycogen calculated from the determination in white blood cells is lower than that determined in whole blood, under the assumption that the leucocytes represent the only glycogen-containing constituent of blood.

<sup>10</sup> The analysis of plasma was carried out on 2 cc.; that in the packed red blood cells on 180 to 200 mg. following the procedure on white blood cells.



## ACKNOWLEDGMENT

I acknowledge with gratitude my indebtedness to Dr. Gerhard Schmidt for his valuable advice.

## SUMMARY AND CONCLUSIONS

The amounts of glycogen in blood and in its various constituents have been determined. The analysis of glycogen in blood requires certain modifications of the usual technique and special precautions which have been described in detail.

The average of the glycogen content of normal human whole blood is 5.5 mg./100 cc. The average of the glycogen content per million normal white blood cells is 2.54  $\gamma$ . Blood platelets do not contain a measurable amount of glycogen. Evidence is presented supporting the assumption that the reducing substances found in hydrolyzates of platelets originate mainly from ribonucleic acid. Plasma and red blood cells were found to be free of glycogen. The granulated leucocyte is apparently the only carrier of glycogen in blood.

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# Precipitation of Riboflavin from Aqueous Solution by Bacteriological Reduction

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## INTRODUCTION

Riboflavin is the only vitamin, insofar as present knowledge goes, which is capable of being synthesized by microorganisms in liquid media to the extent of 200–300 or more  $\gamma$ /ml. Such levels have been produced, for example, by yeasts of the *Candida* genus (1), by *Ashbya gossypii* Guilliermond (2) and by *Eremothecium ashbyii* Guilliermond (3) under appropriate conditions.

The ability of *E. ashbyii* to synthesize riboflavin has been studied fairly extensively. The recent work of Schopfer (4) describes many of the nutritional requirements of the organism as related to riboflavin production. A summary of literature describing *E. ashbyii* accompanies this article. Quite recently Renaud and Lachaux (5) were able to obtain 159  $\gamma$  of riboflavin/ml. in 24 days by the use of *E. ashbyii*. However, the ability of this organism to produce concentrations of riboflavin in liquid media of over 200  $\gamma$ /ml. has not generally been recognized. Lower levels, generally less than 100  $\gamma$ /ml., have been produced by organisms such as *Clostridium acetobutylicum* (6, 7, 8).

Numerous chemical methods have been employed for riboflavin recovery from biological substrates (9) most of which involve adsorption-elution techniques. These methods are somewhat cumbersome in comparison to the methods described by Hines (10, 11) and modified by McMillan (12). Hines' basic method (10) involved the precipitation of a reduced form of riboflavin by microbiological control of the reducing ability of the solution. Approximately 90% of the riboflavin contained in a solution of 200–300  $\gamma$ /ml., for example, may be precipitated as an undefined, amorphous reduced structure by the anaerobic metabolic action of certain bacteria, particularly a group of generally avirulent streptococci. Some specific bacteria capable of achieving the precipitation are *Streptococcus faecalis*, *S. liquefaciens*, *S. cremoris*, *S. zymogenes*, *S. lactis*, *Escherichia coli*, *Serratia plymuthensis*, and some Clostridia. A minimum riboflavin concentration of about 65  $\gamma$ /ml. is generally required for bacteriological precipitation or riboflavin. The fermentative action results in the attaining of an Eh of about – 100 millivolts, or below, with a final pH value of about 4.5.

Subsequently, it was found (11) that an operative oxidation-reduction condition could be produced by strictly chemical reductive means. Upon addition of an appropriate reducing agent, the precipitation of a reduced riboflavin structure occurred almost immediately in contrast to a requirement of about 4 or more hours to start precipitation bacteriologically. Some of the effective chemical reducing agents were sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ ), stannous, titanous, chromous and vanadous salts.

Investigations to find bacteria capable of precipitating riboflavin from aqueous solutions had been undertaken to arrive at an industrially operative process. There were many organisms which did not cause this precipitation from the media employed under the experimental conditions. Among these organisms were some strains of *Staphylococcus aureus* and *Staph. albus*. There should undoubtedly be extenuating circumstances or altered conditions under which some of these non-precipitating organisms will cause precipitation by reduction. Since it was found shortly after the bacteriological studies had begun that a chemical reduction method could be employed to advantage, efforts were ultimately concentrated along this line.

The first observations that a riboflavin structure could be precipitated from aqueous biological media in a state of reasonable purity (80-90%) were made by Hines in these laboratories. He had been studying the production of riboflavin by *Eremothecium ashbyii*. In some filtered media containing over 100  $\gamma$ /ml. of riboflavin which had stood at room temperature for about 24 hours, he noted that the solution, which had originally been a clear, fluorescent yellow-green, had been largely decolorized and a considerable orange-red precipitate had formed. Investigation immediately showed that this precipitate was indeed a form of riboflavin which dissolved readily in water with a moderate amount of aeration. The purity of riboflavin in the precipitate approached 90%. Further study showed that the agent responsible for the precipitation was *Streptococcus faecalis*. The method was obviously relatively practical for commercial riboflavin recovery from biological and perhaps other media. This form of riboflavin can be readily converted to pure, crystalline riboflavin.

The purpose of this paper is to present additional information on the bacteriological precipitation of riboflavin from aqueous media.

## EXPERIMENTAL

### 1. Analytical Methods

Riboflavin was determined fluorophotometrically on filtered and appropriately diluted aqueous solutions at or near pH 6 by means of the Pfaltz and Bauer or Coleman fluorophotometers. The riboflavin precipitates were separated from the mother liquor by centrifugation. Samples of the precipitate were dissolved in distilled water or in dilute pH 6 phosphate buffer by vigorous agitation with air in order to oxidize the reduced riboflavin to the normal oxidized form and thereby to dissolve it. Reduced riboflavin solutions were also aerated prior to fluorophotometric analysis.

The oxidation-reduction potential of a medium was measured using a Coleman pH Electrometer, Model 3D, employing a platinum electrode and standard half-cell. This instrument was also employed for pH measurements employing a glass electrode. The relationship of biological metabolism to redox potential has been described

extensively by Hewitt (13). It should be kept in mind that the oxidizing or reducing power or condition of a medium is a function of both pH and Eh. The term, Eh, as employed in this paper signifies single potential of the redox (platinum) electrode in millivolts.

The instrument was checked for redox accuracy as suggested by Hewitt (13). This method involved measurement of the  $E_h^0$  of quinhydrone in potassium acid phthalate buffer and in  $N/10$  HCl. The  $E_h^0$  value of quinhydrone in phthalate buffer at 20°C. should be + 473 mv., while the value of quinhydrone in  $N/10$  HCl should be + 642 mv.

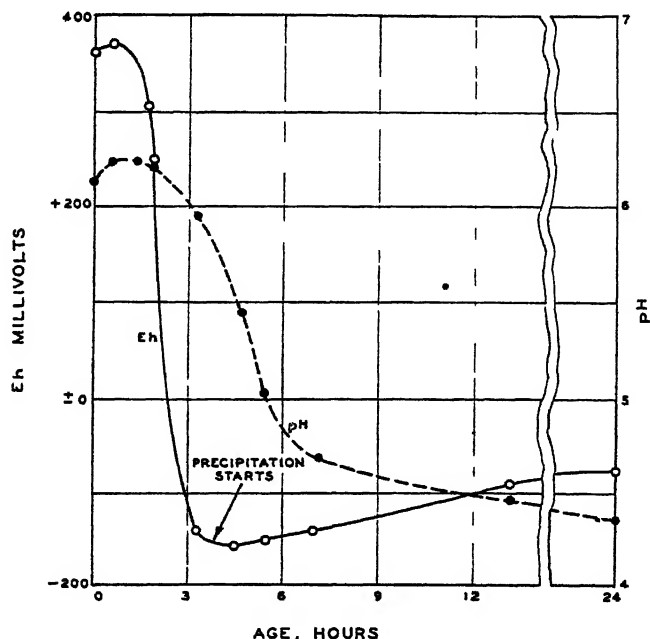


FIG. 1

The pH and Eh Conditions during a Typical Riboflavin Precipitation  
by *Streptococcus faecalis*

Medium: *E. ashbyii* filtrate at 283  $\gamma$  of riboflavin/ml.

Inoculum: 1% *Streptococcus faecalis* grown in *E. ashbyii* filtrate.

Incubation: Static at 28–29°C.

## 2. Characteristic Bacteriological Precipitation of Riboflavin

The media of particular interest from which it was desired that riboflavin be recovered in these laboratories were the solutions resulting from the action of *Eremothecium ashbyii* on a number of substrates

(3, 10). The riboflavin concentration in the filtered liquor assayed 200–300 or more  $\gamma$  of riboflavin/ml., and was a fluorescent yellow-green in color.

Fig. 1 shows some of the characteristic Eh and pH data during precipitation of riboflavin from a filtrate of an *E. ashbyii* biosynthesis by the reducing action of a strain of *Streptococcus faecalis*. The final riboflavin concentration in solution after precipitation was 27  $\gamma$ /ml. The precipitate contained about 90% of the original riboflavin in solution.

During the incubation, it was observed that the color of the medium became lighter as time progressed until the red-orange precipitate started forming at about 4½ hours. It has been observed that sometimes the first precipitate which formed was green, but this has been only transient; the red-orange colored product finally resulted. The green precipitate usually existed when the Eh dropped more slowly than usual, and a less negative Eh level was produced. Even under such conditions, the green precipitate was eventually converted to red-orange.

A number of filtrates have been obtained from *E. ashbyii* biosyntheses in substrates of suboptimum nutrient levels. Many of these did not yield riboflavin-containing precipitates when inoculated with appropriate streptococci, or the quantity of riboflavin precipitated was relatively low. The addition of nutrients, particularly glucose, then promoted precipitation. The addition of  $\text{KH}_2\text{PO}_4$  on the other hand decreased slightly the quantity of riboflavin precipitated.

Riboflavin produced by *Clostridium acetobutylicum* (6, 7) has been precipitated by *S. faecalis*, as well as the riboflavin from artificially prepared media which employed crystalline riboflavin.

### 3. The Effect of Temperature and pH on Precipitation

Using the nutritionally adequate *E. ashbyii* filtrate previously described for a basal medium, and with a riboflavin assay of 310  $\gamma$ /ml., the effects of temperature and initial pH on riboflavin precipitation by *S. faecalis* are shown in Fig. 2. A drop of pH to the range of about 4–5 was regularly found during these fermentations.

The results indicate an improved percentage riboflavin precipitation at lower operative temperatures under the experimental conditions. A reduced solubility of the precipitate might be expected at reduced

temperatures. Lower temperatures exhibit retarded biological activity as indicated at 5°C. with no precipitation, and at 16°C. with delayed precipitation at less optimum initial pH levels.

Another experiment indicated that at 7–10°C. no precipitation was evident until after about 3 days incubation. The optimum initial pH

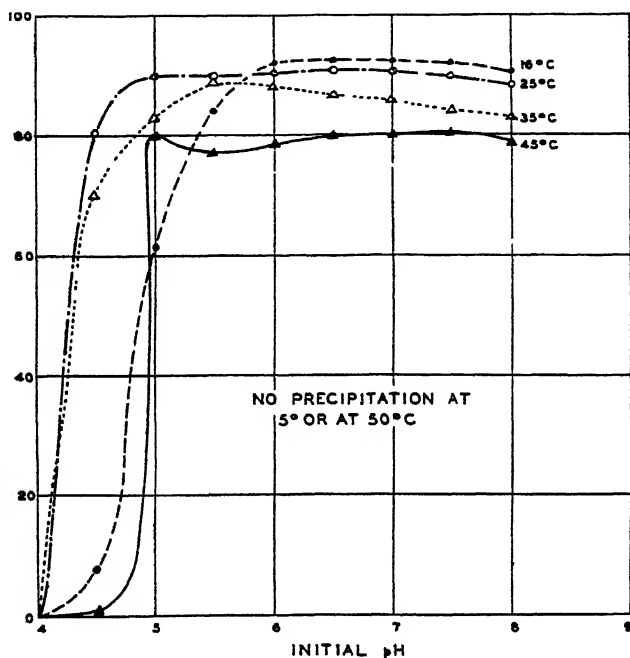


FIG. 2

The Effect of Temperature and Initial pH on Riboflavin Precipitation  
by *Streptococcus faecalis*

Medium: *E. ashbyii* filtrate at 310  $\gamma$  of riboflavin/ml.

Inoculum: 1% *Streptococcus faecalis* grown in *E. ashbyii* filtrate.

Incubation: 18 hours at the temperatures indicated.

range was shown to be about 6–7.5, and the optimum temperature was about 25°C. Losses have been observed when the media were appreciably more alkaline than 8. Riboflavin is increasingly unstable at higher pH levels.

#### 4. Effect of Aeration on Riboflavin Precipitation

In order to show further that a reducing potential is essential for riboflavin precipitation, a series of flasks of inoculated medium was aerated by shaking for various periods followed by static incubation as indicated in Table I. It was observed that no riboflavin precipitation

TABLE I  
*The Effect of Aeration for Various Periods on Riboflavin Precipitation  
by Streptococcus faecalis*

Medium: *E. ashbyii* filtrate at 280  $\gamma$ /ml., pH = 6.0, 150 ml. in 1 liter flasks.

Inoculum: 1% of a 22 hour liquid culture of *S. faecalis*.

Incubation: 28 hours at 30°C. with mechanical shaking for aeration at the beginning of the incubation for the time indicated.

Aeration period	Static period	Final pH	Final Eh	Mother liquor	Apparent precipitation per cent by difference <sup>a</sup>	Visual precipitation
<i>hrs.</i>	<i>hrs.</i>		<i>mV</i>	$\gamma$ /ml.		
4	24	4.49	- 31	28	90	+
8	20	4.52	- 41	26	91	+
12	16	4.92	- 47	47	83	+
16	12	4.95	+ 356	249	0	-
20	8	4.96	+ 345	233	0	-
24	4	5.00	+ 328	243	0	-
28	0	5.03	+ 294	248	0	-

<sup>a</sup> These values are based on the 280  $\gamma$ /ml. original value, except for the zero values which were so designated simply because there was, in fact, no precipitate. A loss of about 30  $\gamma$ /ml. during the process would show an unreal apparent precipitation of about 12%.

<sup>b</sup> Note that this is the final and not the lowest Eh of media which have a considerable surface exposed to air.

occurred during aeration. It was also found that, if aeration occurred for longer than about 12 hours prior to standing, the Eh value at 28 hours was quite positive with no precipitation of riboflavin. When aeration occurred for 12 hours or less, the final Eh value was moderately negative and precipitation occurred. Apparently the nutrients of the media had been largely exhausted after about 16 hours. This evidence, along with the fact that chemical reducing agents (11, 12) will bring about the precipitation of riboflavin, indicates that a negative single electrode potential for the medium is of prime importance for precipitation to occur.

## DISCUSSION

The phenomenon of bacteriological precipitation of an undefined reduced form of riboflavin was an unpredicted observation. That proper redox conditions, and not a specific enzyme action, are responsible for the precipitation is indicated by the facts that:

- (1) Precipitation does not occur unless the measured Eh value is in the proper reducing range. Aeration inhibits precipitation.
- (2) Precipitation can be brought about by the action of certain chemical reducing agents on riboflavin solutions.

It has been observed that sometimes during bacteriological reduction of the riboflavin-containing media, the first precipitate which forms is rather deep green in color. Within a short time, generally 30 minutes or less, the green precipitate already formed and all subsequent precipitate has always been converted to the typical red-orange colored form. It might be possible under critically controlled conditions to precipitate and recover the green form. When the final precipitate is separated by centrifugation, the red-orange putty-like mass, upon exposure to air, becomes green on the surface temporarily. On further oxidation the green form is converted to the final orange-yellow oxidized form. This crude riboflavin can be purified to obtain crystalline riboflavin.

The occurrence of at least three different forms of riboflavin in various stages of reduction during the *S. faecalis* precipitation process is indicated. They are (1) the initial oxidized form, (2) the intermediate, transient green precipitate, which may be perhaps a semiquinone, and (3) the final reduced red-orange precipitate.

The existence of riboflavin structures of intermediate stages of reduction has been shown by Kuhn and Wagner-Jauregg (14), by Michaelis, Schubert and Smythe (15) and by Kuhn and Ströbele (16). A red reduced form was obtained (14) in very strongly acid solutions. At higher pH values, 2-12, Michaelis *et al.* (15) showed the presence of a green intermediate form. Kuhn and Ströbele (16) obtained, in the crystalline state, a green structure called verdoflavin at an oxidation level corresponding to one riboflavin for one monohydroflavin radical. In addition, they obtained the red rhodoflavin as a hydrochloride composed of one dihydroflavin for one monohydroflavin. Chloroflavin, another green form, was presumably a compound intermediate between verdoflavin and rhodoflavin, but which possessed an abnormally low paramagnetic susceptibility. Michaelis and Schwartzenbach (17) demonstrated that, of all these forms, only the free radical can exist in dilute aqueous riboflavin solutions.



It was indicated (18) that at higher concentrations "such as never occur physiologically" the free radical could exist in equilibrium with a dimer, but no other forms would be present. In connection with physiologically occurring quantities of riboflavin, Guilliermond (19) showed that riboflavin was synthesized by *Eremothecium ashbyii* to such an extent that it crystallized in the vacuoles.

It was found by Haas (18, 20) that, when the yellow respiratory enzyme (riboflavin phosphate ester attached to a specific protein) was reduced at 0°C. by the reduced triphosphopyridine nucleotide (coenzyme), a red intermediate formed which possessed an absorption spectrum which was the same as that of Kuhn's rhodoflavin which was produced under extremely acid conditions. Thus a red structure was produced under more nearly neutral conditions rather than under the strongly acid conditions previously described. This was presumably a flavin-protein-nucleotide.

The riboflavin structure described in this paper is a red-orange, amorphous precipitate which is generally produced within the pH range of about 4.5-7. It is not a pure riboflavin structure; analyses show about 85-90% riboflavin. Considerable of the remaining 10-15% is inorganic, including appreciable phosphate. Heat-sterilized *E. ashbyii* filtrates, which should be free of the flavoprotein because of the action of heat, yield a red-orange precipitate upon addition of reducing agents such as  $\text{Na}_2\text{S}_2\text{O}_4$  at near neutral conditions. Thus a red-orange precipitate was produced apparently non-enzymatically in contrast to Haas' (20) method, but under similar pH conditions.

It is evident from the bacteriological data presented that both Eh and pH have been variable concurrently. It is rather difficult to hold one of these values constant in experiments such as these while allowing the other to vary. It would undoubtedly be possible to hold the pH constant automatically by means of one of the commercial glass electrode control instruments. It is also possible to maintain a constant oxidation-reduction potential in bacteriological media according to the method of Hanke and Katz (21). We have not attempted either of these control methods as yet, however.

It should be quite possible that some of the riboflavin analogues will also undergo this type of precipitation.

#### SUMMARY

Supplementary data have been presented which describe the precipitation of reduced forms of riboflavin from certain nutrient media by the metabolic reducing action of *Streptococcus faecalis* under anaerobic conditions.

A measured single electrode (platinum) potential, or Eh, of about - 100 millivolts or lower is necessary for good precipitation.

Three forms of riboflavin are involved in the precipitation: (1) the original oxidized form in solution, (2) the intermediate, evanescent, green precipitate form, and (3) the final red-orange reduced precipitate.

Up to about 90% of the dissolved riboflavin can be precipitated from solution by appropriate bacteriological reduction.

The red-orange precipitate herein described was obtained under different conditions than were employed for Kuhn's rhodoflavin and for Haas' red form.

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# Oxalacetic and Pyruvic Carboxylases in Some Dicotyledonous Plants\*

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## INTRODUCTION

Recent work in this and other laboratories (1, 2, 3, 4) has indicated that the enzyme oxalacetic carboxylase is intimately associated with the fixation of carbon dioxide by bacteria and by animal tissues. In this connection we have undertaken an examination of the tissues of higher plants, with the object of discovering other oxalacetic carboxylases and thereby adding to an understanding of the general significance of the reaction.

A survey of the literature revealed only limited information on the distribution of ketoacid carboxylases in higher plants, most investigations having been restricted to the  $\alpha$ -keto carboxylase which attacks pyruvic acid. This enzyme has been demonstrated directly by modern methods in legumes (5), cereal grains (6, 7) and tomato seedlings (7).<sup>1</sup> Furthermore, indirect evidence for the existence of pyruvic carboxylase in the tissues of higher plants has been provided by the demonstration of the widespread occurrence of alcohol formation (9, 10, 11, 12). The action of these pyruvic carboxylases on oxalacetic acid has not been examined, however, nor has the occurrence of a specific oxalacetic carboxylase in higher plants been reported.

We have accordingly made a broad though necessarily incomplete survey of the occurrence of directly demonstrable carboxylase activity in a variety of accessible dicotyledonous plant materials. The substrates used were limited to oxalacetic acid and its breakdown product, pyruvic acid. Because of the spontaneous decomposition of oxalacetate to pyruvate, it is not possible to make a proper evaluation of the direct

\* This work was supported in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

<sup>1</sup> See (8) for earlier literature, which includes observations on other species.

effect of a given preparation on oxalacetate unless the effect of the preparation on pyruvate is also known.

This survey has revealed that the crystalline globulins of pumpkin seeds and squash seeds exhibit specific oxalacetic carboxylase activity. An extensive addition has also been made to the list of plants shown to contain pyruvic carboxylase. This enzyme has been found widely distributed in the family *Cucurbitaceae*, which include melons and cucumbers as well as pumpkins and squashes, in the *Cruciferae* (cabbage and related varieties, radish, turnip) and the *Umbelliferae* (carrot, parsley, parsnip). The action of a number of representative pyruvic carboxylase preparations on oxalacetate has been examined with the object of obtaining information on their ability to decarboxylate this compound. Several sources, notably parsley root, parsnip and black radish, have been shown to contain a heat-labile factor which attacks oxalacetate directly. In the case of the parsley root, which has been examined in more detail, the heat-labile factor attacking oxalacetate directly has been shown to be independent of pyruvic carboxylase.

## EXPERIMENTAL

### *Methods and Preparations*

Oxalacetic acid and pyruvic acid were prepared by methods previously employed (4). Seeds<sup>2</sup> and fresh plant material were bought on the market.

For the preparation of materials for testing, a standard procedure was adopted. All plant tissues were thoroughly cleaned and prepared for assay by grinding through a meat grinder and/or in a mortar with sand, depending on the procedure required to produce a fine state of subdivision. Water was added in small quantities as indicated in the text only when necessary to produce a suspension which could be pipetted easily. Coarse particles were strained off through muslin but no centrifugation was done unless specified. The material tested consisted, therefore, of a ground suspension containing all constituents of the original tissues except those which were highly resistant to subdivision by grinding. Preparations were made at room temperature and examined immediately.

The test system was designed for convenience, and consisted of the measurement of liberated carbon dioxide by the usual Warburg manometric technique. One ml. plant preparation was used unless otherwise indicated. The reaction mixture was buffered with 0.125 M acetate at pH 5.0, under which condition there is no retention of bicarbonate by the medium. The water bath was kept at 30°C. and air was used

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<sup>2</sup> Named varieties of cucurbit seeds were purchased from Vaughan's Seed Store, 10 W. Randolph St., Chicago. When seeds were harvested from fresh plants, this is so indicated in the text and tables. In these latter cases the conclusions do not, of course, involve any precise knowledge of genetic constitution, since some of the plants examined are known to be susceptible to a wide range of cross pollination.

as the gas phase. Substrates were tipped in from the side arm after equilibration. Pyruvate was added as 0.1 ml. of 0.1 *M* solution of the sodium salt. Oxalacetate was added as 0.1 ml. of a solution containing about 1 mg. of the free acid, unless otherwise specified. This solution was always prepared immediately before use. In every case, both  $O_2$  consumption and  $CO_2$  evolution were determined, the former directly, with alkali in the center pot, the latter after correction for the measured oxygen consumption. Blanks on  $CO_2$  evolution and oxygen consumption in the absence of added substrate were always determined also, and results were evaluated in terms of the differences observed with and without added substrate.

In view of our ignorance of the properties of the enzymes sought, there is no assurance that the testing conditions employed are necessarily the best for the demonstration of a particular activity. Furthermore, no special precautions were taken to prevent inactivation during preparation. Negative results cannot be taken as adequate evidence for the absence of carboxylase but simply indicate that the enzyme cannot be demonstrated by the chosen procedure.

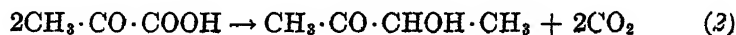
### Nomenclature

A precise experimental definition of the terms "pyruvic carboxylase" and "oxalacetic carboxylase" as employed in this paper is presented in the following sections. These definitions correspond to those established by previous usage. Although there is no intention of suggesting a change in this terminology, it should be pointed out that pyruvic carboxylase might more precisely be called an  $\alpha$ -(de)carboxylase and that oxalacetic carboxylase is a  $\beta$ -(de)carboxylase.

### Criteria Employed for Determining the Presence of Pyruvic Carboxylases

The presence of pyruvic carboxylase was regarded as established in any preparation which caused an evolution of one mole of  $CO_2$  per mole of added pyruvate without simultaneous extra oxygen consumption. Rates below five  $\mu l.$ /hour were regarded as inconclusive and listed as negative. Rates above five  $\mu l.$ /hour but below forty  $\mu l.$  in the first hour were regarded as positive, even though they were not run to completion and the equivalence of  $CO_2$  formed to pyruvate which disappeared was not demonstrated. No preparations were found which possessed any ability to decarboxylate pyruvate after heat-inactivation (boiling water bath for 2 minutes).

The pyruvic carboxylase so defined may catalyze reaction (1) and/or reaction (2) or similar unknown condensations.



*Criteria Employed for Determining the Presence of Oxalacetic Carboxylase*

The demonstration of the presence of oxalacetic  $\beta$ -carboxylase can best be considered separately, first for the case where pyruvic carboxylase is absent or very weak, and second, for the case where pyruvic carboxylase is present.

In the absence of pyruvic carboxylase the main difficulty is the occurrence of a spontaneous  $\beta$ -decarboxylation according to equation (3).



This reaction is catalyzed by small amounts of several multivalent cations (13) and by heat-coagulated tissue preparations (14). The catalytic effect of a tissue preparation on reaction (3) is probably due to a variety of substances present and cannot be considered enzymic unless other criteria are employed. We have chosen the following conditions as criteria for the presence of oxalacetic  $\beta$ -carboxylase in the absence of pyruvic carboxylase.

a. The activity is heat-labile. In other words, the rate of  $\text{CO}_2$  evolution from oxalacetate is greater in the presence of an unheated preparation than in the presence of an equivalent amount of a preparation held in a boiling water bath for two minutes.

b. The heat-labile factor is a large molecule (non-dialyzable), although a dialyzable cofactor may be necessary. This latter criterion is added in order to preclude the application of the term enzyme to unknown organic substances of low molecular weight which might be destroyed on heating.

c. The products of the reaction are pyruvate and  $\text{CO}_2$  formed in amounts equivalent to the oxalacetate added.

It can be shown that many preparations satisfy the first of these criteria if small differences between rates of  $\text{CO}_2$  evolution from heated and unheated preparations (usually 10–30%) are regarded as significant. There are technical reasons, however, for questioning such results. The formation of a heavy coagulum, for example, may prevent contact of the reacting substances. Since these factors have not been investigated in detail, we have limited our present report to that group of reactions in which more than 50% of the catalytic action was lost on heating.

The difficulty of demonstrating a direct enzymic decarboxylation of oxalacetate is enhanced in the presence of pyruvic carboxylase, since the latter enzyme can further decarboxylate the pyruvic acid formed by nonenzymic  $\beta$ -decarboxylation. In accordance with expectations, all preparations which contained pyruvic carboxylase in sufficient

amounts, caused the liberation of two moles of  $\text{CO}_2$  from one mole of oxalacetate. As indicated by Werkman and Wood (15), it is probable that such reactions proceed in two steps, but there is a question as to whether the initial decarboxylation removes the carbon dioxide  $\alpha$  or  $\beta$  to the carbonyl group. Thus, the reaction may follow either, or perhaps even both, of the following paths:

- (a) Oxalacetate  $\rightarrow$  pyruvate +  $\text{CO}_2$   
Pyruvate  $\rightarrow$  acetaldehyde +  $\text{CO}_2$
- (b) Oxalacetate  $\rightarrow$  malonic semialdehyde +  $\text{CO}_2$   
Malonic semialdehyde  $\rightarrow$  acetaldehyde +  $\text{CO}_2$

It seems very unlikely, however, that pyruvic carboxylase should attack oxalacetate directly to any great extent, in view of its slow action on  $\alpha$ -ketoglutaric acid (16). Reaction sequence (a) must, on the other hand, occur wherever heat-stable catalysts for the  $\beta$ -decarboxylation are present together with an active pyruvic carboxylase.

Determination of the rate of decarboxylation of oxalacetate, before and after heat-inactivation, together with a measurement of the rate of decarboxylation of pyruvate was, therefore, employed as a guide to the possible presence of an oxalacetic carboxylase. More specifically, three rates are measured with the same enzyme preparation:

- (1) The rate of  $\text{CO}_2$  evolution from oxalacetate before heat-inactivation of the enzyme preparation.
- (2) The same, after heat-inactivation of the enzyme preparation.
- (3) The rate of  $\text{CO}_2$  evolution from pyruvate.

If (2) + (3) < (1), the enzyme preparation must attack oxalacetate directly. The significance of results obtained in this fashion will be discussed in more detail in connection with the presentation of the data.

## RESULTS

For convenience of reference, preparations found to contain keto-carboxylases have been listed together in Table I. A list of preparations giving negative results can be found on p. 302.

### *Oxalacetic and Pyruvic Carboxylase in Seeds of the Cucurbits*

An oxalacetic carboxylase was found to be present in the seeds of all pumpkins and squashes which were tested. The initial preparations usually contained pyruvic carboxylase likewise, but the latter was always of low activity relative to the former. Furthermore, the pyruvic



carboxylase could be easily removed or inactivated without any decrease in the activity of the oxalacetic carboxylase.

Twenty varieties, representing the three species, *Cucurbita maxima*, *Cucurbita moschata* and *Cucurbita pepo*, were tested and results obtained with all were almost identical with respect to the action on oxalacetate, although some variation was observed with pyruvate. Experiments with *Cucurbita maxima*, variety Hubbard, and *Cucurbita pepo*, variety Zucchini Dark Green, have been graphed in Fig. 1 and Fig. 2, respectively, to illustrate the nature of the results obtained in the initial test system. The Hubbard squash seeds contained the average amount of pyruvic carboxylase; the Zucchini squash seeds contained the largest amount found in any of the squashes and pumpkins tested. Both

TABLE I  
*Preparations Containing Ketoacid Carboxylases*

Family	Species	Common name	Part of plant used and preparation of material
<i>Cucurbitaceae</i>	<i>Cucurbita maxima</i> Duchesne.	Squashes and Pumpkins	Seeds—testa removed seeds ground to fine powder and suspended in 5 volumes water. Pulp (parenchyma of the mesocarp) ground and strained through gauze.
	<i>Cucurbita moschata</i> Duchesne.		
	<i>Cucurbita pepo</i> L.		
	<i>Citrullus vulgaris</i> Schrad.	Watermelon	Seeds and pulp prepared as with <i>Cucurbita</i> .
	<i>Cucumis melo</i> L.	Muskmelon	Seeds and pulp prepared as with <i>Cucurbita</i> .
	<i>Cucumis melo</i> L.	Honeydew melon	Seeds and pulp prepared as with <i>Cucurbita</i> .
	<i>Cucumis sativus</i> L.	Cucumber	Seeds and pulp prepared as with <i>Cucurbita</i> .
<i>Leguminosae</i>	<i>Pisum sativum</i> L.	Green peas	Fresh seeds, removed from pods, ground with sand and juice expressed through muslin.
	<i>Phaseolus lunatus</i> L.	Green lima beans	As above—but one-half volume water added during grinding.
	<i>Glycine Soja</i> Sieb.	Soy bean	Purchased seeds, treated as above, but 5 volumes water added during grinding.

TABLE I (Continued)

Family	Species	Common name	Part of plant used and preparation of material
<i>Cruciferae</i>	<i>Brassica oleracea</i> var. <i>capitata</i> L.	Cabbage	Whole head ground fine and juice expressed. Stalk and leaves also prepared separately in same manner.
	<i>Brassica oleracea</i> var. <i>gemmifera</i> DC.	Brussels sprouts	Buds ground fine and juice expressed.
	<i>Brassica oleracea</i> var. <i>botrytis</i> L.	Broccoli	Stalk and inflorescence ground fine, juice expressed.
	<i>Brassica oleracea</i> var. <i>botrytis</i> L.	Cauliflower	Inflorescence ground fine, juice expressed.
	<i>Brassica oleracea</i> var. <i>CaULO-Rapa</i> DC.	Kohlrabi	Enlarged stem ground fine and juice expressed. Leaves treated similarly.
	<i>Brassica rapa</i> L.	White turnip	Enlarged hypocotyl and root ground fine and juice expressed.
	<i>Brassica campestris</i> var. <i>Napo-Brassica</i> DC.	Yellow turnip (rutabaga)	Root ground fine and juice expressed.
	<i>Radicula armoracia</i> Robins.	Horseradish	Root ground fine and juice expressed.
	<i>Raphanus sativus</i> L.	Radish (Red, white and black varieties)	Root ground fine and juice expressed.
<i>Umbelliferae</i>	<i>Daucus carota</i> L.	Carrot	Root ground fine and juice expressed.
	<i>Pastinaca sativa</i> L.	Parsnip	Root ground fine with half volume water and juice expressed.
	<i>Petroselinum hortense</i> Hoffm.	Parsley	Root ground fine and juice expressed.

preparations show essentially similar properties, however. The milky suspension from the ground seeds causes a rapid evolution of  $\text{CO}_2$  from added oxalacetate. When the amount of  $\text{CO}_2$  evolved is equivalent to the oxalacetate added, the rate of  $\text{CO}_2$  evolution diminishes abruptly and reaches the value observed with added pyruvate alone.

The heat-inactivated preparation also causes an evolution of  $\text{CO}_2$  from oxalacetate, but it is apparent from the figures that a considerable

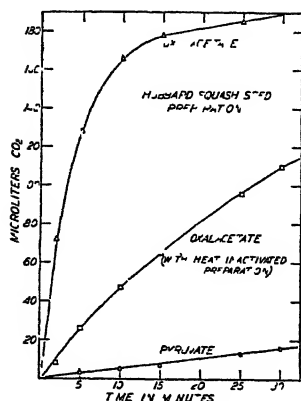


FIG. 1

### The Action of a Hubbard Squash Seed Preparation on Oxalacetate and Pyruvate

One ml. suspension, 0.5 ml. 0.5 *M* acetate buffer, pH 5.0. Total volume made up with water to 2.0 ml. 1 mg. (170  $\mu$ l.) oxalacetic acid or 224  $\mu$ l. sodium pyruvate tipped in at time 0.  $t = 30^\circ\text{C}$ . Gas phase, air. Heat inactivated preparation was held in boiling water bath for two minutes before other additions were made.

portion of the oxalacetic carboxylase action is heat-labile. It is likewise apparent that the preparations must contain a heat-labile factor which attacks oxalacetate directly, since the rate of pyruvate decarboxylation is far too slow to account for more than a small fraction of the decrease in the rate of CO<sub>2</sub> evolution from oxalacetate after heat-inactivation.

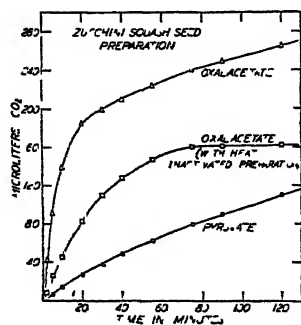


FIG. 2

### The Action of a Zucchini Dark Green Squash Seed Preparation on Oxalacetate and Pyruvate

Conditions as in Fig. 1.

Seeds of other members of the *Cucurbitaceae*, melons and cucumbers, were likewise usually found to contain pyruvic carboxylase but evidence for a factor acting directly on oxalacetate was questionable or lacking.

Representative results obtained with all cucurbit species tested are listed in Table II. All experiments were conducted in the same manner as those graphed in Figs. 1 and 2. To give a basis of comparison, the data on the Zucchini Dark Green squash (Fig. 2) have been included in the Table.

In general, the squash and pumpkin seeds differed in their action on oxalacetate only insofar as the presence of varying amounts of pyruvic carboxylase influenced the results. With watermelon, muskmelon, cucumber and honeydew melon seeds, a decrease in  $\text{CO}_2$  evolution from oxalacetate was observed after heating but it was considerably smaller than that obtained with squash and pumpkin. The former results must at present be regarded as of questionable significance insofar as evidence for the existence of an oxalacetic carboxylase is concerned.

The occurrence of pyruvic carboxylase is seen in Table II to vary considerably in all species of cucurbits, both in pumpkins and squashes as well as in melons and cucumbers, in contrast to the uniform occurrence of oxalacetic carboxylase in the pumpkins and squashes. Table III shows the action on pyruvate of all the varieties of squashes and pumpkins tested. (The results obtained with the preparations in Table III and oxalacetate are not listed because of their similarity to the representatives of each of the species given in Table II.) There is obviously no uniformity among varieties within a given species, nor do the data indicate whether a certain pyruvic carboxylase content is characteristic of a given variety.

Further experiments indicated that seeds recently removed from fresh plants generally contained larger amounts of pyruvic carboxylase than purchased seeds. Thus watermelon, muskmelon and cucumber seeds harvested directly from the fruit contained pyruvic carboxylase in an amount approximately equal to that found in honeydew melon seeds (Table II) although the purchased cucumber and muskmelon seeds were found to contain no activity, as indicated in the table. When, however, these same muskmelon seeds were soaked in water, pyruvic carboxylase activity appeared. In the standard test, seeds soaked three hours gave 34  $\mu\text{l. CO}_2$  from pyruvate in one hour; seeds soaked twenty-two hours gave 133  $\mu\text{l. CO}_2$  from pyruvate in the same

TABLE II  
Carbon Dioxide Evolved from Oxalacetate and Pyruvate  
in the Presence of Cucurbit Seed Suspensions<sup>+</sup>

Species	Time	Oxalacetate		Pyruvate
		Unheated	Heated	
	<i>minutes</i>	$\mu\text{l. CO}_2$	$\mu\text{l. CO}_2$	$\mu\text{l. CO}_2$
<i>Cucurbita maxima</i> , var. Vaughan's Chicago Warted Hubbard Squash	5	125	26	4
	10	140	42	7
	15	148	59	9
	20	152	74	10
<i>Cucurbita moschata</i> , var. Kentucky Field pumpkin	5	133	33	1
	10	163	59	6
	15	170	79	7
	20	174	95	10
<i>Cucurbita pepo</i> , var. Early White Bush squash	5	108	23	4
	10	156	48	8
	15	177	65	11
	20	188	82	13
<i>Cucurbita pepo</i> , var. Fordhook squash	5	100	25	0
	10	132	43	0
	15	141	59	0
	20	146	73	0
<i>Cucurbita pepo</i> , var. Zucchini Dark Green squash	5	91	26	7
	10	139	46	14
	15	166	66	20
	20	184	83	27
<i>Citrullus vulgaris</i> , var. Ovid watermelon	5	51	23	5
	10	79	42	8
	15	103	59	12
	20	121	74	15
<i>Cucumis melo</i> , var. <i>reticulatus</i> Burrell's Gem muskmelon	5	25	20	0
	10	44	33	0
	15	59	49	
	20	71	61	
<i>Cucumis sativus</i> , var. Fast Green cucumber	5	33	18	
	10	48	29	
	15	64	43	
	20	79	56	0
<i>Cucumis melo</i> Honeydew melon (seeds removed from fresh ripe fruit).	5	78	36	31
	10	110	52	48
	15	143	72	
	20	170	87	83

time. Addition of diphosphothiamine to the inactive preparation made directly from the dry seeds also resulted in the appearance of pyruvic carboxylase activity. These experiments will be reported in detail later, but results already obtained indicate that all cucurbit seeds can be

TABLE III  
*Action of Squash and Pumpkin Seed Suspensions on Pyruvate*

Species	Variety	$\mu\text{l. CO}_2$ from pyruvate in 20'
<i>Cucurbita pepo</i>	Bennings Bush squash	18
	Giant Yellow Summer Crookneck squash	5
	Italian Vegetable Marrow squash	5
	English Vegetable Marrow squash	4
	Sweet Potato Improved squash	8
	Vaughan's Sugar Pie pumpkin	11
	Autumn pumpkin	5
<i>Cucurbita maxima</i>	Banana squash	21
	Boston Marrow squash	10
	Golden Delicious squash	7
	Original Hubbard squash	11
	Mammoth Chili squash	0
<i>Cucurbita moschata</i>	Large Cheese pumpkin	0
	Cushaw Golden pumpkin	0
	White Cushaw pumpkin	3

\* Conditions used were those of the standard test system described in the text. Figures refer to results obtained with 1 ml. of preparation (as described in Table I). The amount of oxalacetate actually added at the time of tipping is always lower than the amount used for making the solution because of the spontaneous decomposition which sets in immediately. Some pyruvate is, therefore, always present. This amount of preformed pyruvate can be measured by decarboxylating the added oxalacetate completely with an enzyme free from pyruvic carboxylase (e.g., pigeon liver oxalacetate carboxylase), then measuring the pyruvate by tipping in yeast carboxylase freed from  $\text{CO}_2$  by evacuation. The amount of pyruvate thus determined was found to exceed the amount of oxalacetate added by about 10  $\mu\text{l.}$  in the usual experiments. This figure will vary, of course, with the time elapsing from the solution of the oxalacetic acid to the tipping. Some variation must, therefore, be expected in the amount of undecomposed oxalacetate added in different experiments, since observation of absolutely accurate timing seemed an unnecessary precaution. Heated and unheated preparations were always run side by side, however, with aliquots of the same oxalacetate solution. No part of the difference observed between them can be attributed to differences in the amount of oxalacetate added.

shown to contain pyruvic carboxylase activity provided tests are conducted on seeds in the proper condition. Results on pyruvic carboxylase given in Tables II and III should not be regarded as typical of a variety but merely as an indication of the type of variations encountered among these members of the same family.

Blanks on  $\text{CO}_2$  production and oxygen consumption in absence of added substrate were virtually negative in the cucurbit seeds. None of these preparations showed any extra oxygen consumption in the presence of the keto acids. The product of the decarboxylation of oxalacetate was pyruvate in every case, as shown by analysis with yeast carboxylase.

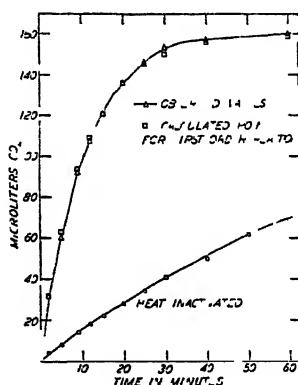


FIG. 3

#### The Action of Dialyzed Water-Insoluble Fraction from Acorn Squash Seeds on Oxalacetate

Conditions as in Fig. 1 except that 0.3 cc. of suspension were used to effect a slower reaction.

#### *The Nature of the Oxalacetic Carboxylase in Squash and Pumpkin Seeds*

An examination of the oxalacetic carboxylase of the acorn squash seeds readily revealed that this activity did not decrease appreciably on dialysis against water and that it was not water-soluble, whereas the pyruvic carboxylase was soluble in water and sensitive to dialysis. When the original suspension was dialyzed against water, centrifuged and the precipitate resuspended in water to the original volume, a preparation was obtained which was free from the pyruvic carboxylase originally present. The experiment shown in Fig. 3 gives results ob-

tained with 0.3 ml. of such a preparation and demonstrates the presence of a heat-labile enzyme which liberates one mole of  $\text{CO}_2$  from one mole of oxalacetate. No pyruvic carboxylase was present. The theoretical amount of pyruvate was formed from oxalacetate. The reaction is apparently monomolecular. The reaction rate constant  $k$  calculated for the interval from 2' to 20' ( $k = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$ ,  $a$  = initial substrate = 160  $\mu\text{l.}$ ,  $x_1 = 32 \mu\text{l.}$   $\text{CO}_2$  evolved at time  $t_1$ ,  $x_2 = 136 \mu\text{l.}$  evolved at time  $t_2$ ) was 0.040. The theoretical points calculated back to the other time intervals are shown on the graph. They fit the experimental curve within the limits or error of the measurements.

Reactions run with 0.1, 0.3 and 0.6 ml. of the same suspension gave  $k$  values of 0.015, 0.040 and 0.074 respectively. No corrections were made for blanks (non-enzymic  $\text{CO}_2$  evolution) in these calculations. In spite of the inaccuracies introduced by omitting such corrections, these figures are taken to indicate that the reaction rate is proportional to the enzyme concentration, as a first approximation.

Further examination of the active precipitate showed that the activity is present in the easily crystallizable globulin which makes up 10–13% of the dry weight of the seeds (17). The enzymic properties of these crystalline cucurbit seed globulins will be reported in detail in another paper.

### *Carboxylases in the Parenchyma of Cucurbitaceous Fruit*

The fresh pulp of all cucurbits which were tested was found to contain pyruvic carboxylase. These preparations were made from the whole fruit after removal of the seeds and the external rind, the main source of the material tested being clearly the fleshy parenchyma of the mesocarp. Observations were limited to acorn squash, pumpkin, cucumber, watermelon, muskmelon and honeydew melon. The squash and pumpkin had low activities (about 40  $\mu\text{l.}$  and 20  $\mu\text{l.}$   $\text{CO}_2$ , respectively, in the first hour), and no further work was done on these preparations.

Of the melons, honey-dew melon and muskmelon gave the most active preparations. The juice which is obtained in large quantities from these fruits can, when fresh, usually decarboxylate 224  $\mu\text{l.}$  pyruvate completely in 1.5 hours. Watermelon and cucumber pulp gave preparations of lower activity, 4–5 hrs. usually being required for the complete decarboxylation of the same amount of pyruvate.



The pyruvic carboxylase preparations from honeydew melon and cucumber pulp were tested with oxalacetate to see whether there was any indication of the presence of a heat-labile factor with direct action on this substrate.

The experiments consisted of a determination of the  $\text{CO}_2$  evolution observed when 224  $\mu\text{l}$ . pyruvate and about 170  $\mu\text{l}$ . oxalacetate (see footnote to Table II) were incubated separately with unheated and with heated preparations of enzymes. Figures are corrected for the small blanks observed without substrate. Identical experiments were performed with most of the pyruvic carboxylase preparations examined and results of representative experiments of this type which are not presented as graphs have been collected in Table IV to facilitate comparison of results obtained with preparations from different sources. All experiments in this table were done under the conditions of the standard test system with corrections made for blanks.

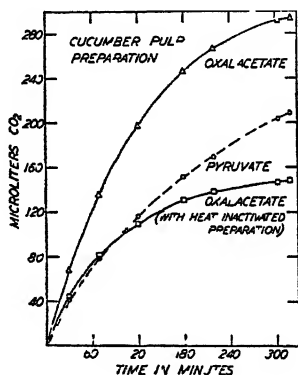


FIG. 4

The Action of a Cucumber Pulp Preparation on Oxalacetate and Pyruvate  
Conditions as in Fig. 1.

Fig. 4 shows that cucumber pulp does not lose more than half its ability to decarboxylate oxalacetate when it is heated. There is, therefore, no clear cut evidence for the presence here of a true oxalacetate carboxylase. Thus, in 2 hours 110  $\mu\text{l}$ .  $\text{CO}_2$  were evolved from oxalacetate added to the heat inactivated preparation which had no action on pyruvate. From the unheated preparation, 197  $\mu\text{l}$ .  $\text{CO}_2$  were evolved from oxalacetate in the same amount of time. The bulk of the extra  $\text{CO}_2$  evolution, observed in the presence of the active pyruvic carboxylase, might reasonably be expected to be formed by decarboxylation of pyruvate formed non-enzymically, since the preparation gave

TABLE IV  
*Carbon Dioxide Evolved from Oxalacetate and Pyruvate  
 Incubated with Plant Preparations\**

Source	Time	Oxalacetate		Pyruvate
		Unheated	Heated	
	<i>minutes</i>	$\mu\text{l. CO}_2$	$\mu\text{l. CO}_2$	$\mu\text{l. CO}_2$
Honeydew melon pulp (parenchyma of the mesocarp)	10	33	19	35
	20	64	34	63
	40	117	60	109
	120	244	129	227
Soy beans (ground with five volumes water)	10	59	25	112
	20	109	48	195
	40	175	86	226
	120	298	150	226
Brussels sprouts	10	73	59	22
	20	125	99	44
	40	190	143	80
	120	288	180	184
Broccoli	10	51	40	24
	20	95	70	49
	40	160	111	90
	120	267	164	194
Cabbage (stem)	10	121	52	85
	20	209	90	157
	40	308	126	227
	120	349	149	238
Cabbage (leaves)	10	47	38	0
	20	84	68	6
	40	126	107	17
	120	185	148	51
White turnip	10	28	20	20
	20	57	35	38
	40	114	58	72
	120	244	112	178

TABLE IV (Continued)

Source	Time	Oxalacetate		Pyruvate
		Unheated	Heated	
	<i>minutes</i>	$\mu\text{l. CO}_2$	$\mu\text{l. CO}_2$	$\mu\text{l. CO}_2$
Yellow turnip	10	55	29	38
	20	98	53	78
	40	161	83	142
	120	278	134	242
Horseradish	10	97	60	74
	20	173	101	135
	40	260	138	224
	120	306	164	237
Red radish	10	51	21	48
	20	96	36	83
	40	166	63	140
	120	292	120	236
Carrot	10	43	18	43
	20	83	34	77
	40	149	59	129
	120	295	124	230
Parsnip (ground with one-half volume water)	10	82	25	178
	20	143	45	226
	40	215	76	226
	120	312	135	226
Baker's yeast (Extract of dry powder)	10	34	26	36
	20	64	43	70
	40	118	75	116
	120	247	130	220

\* See footnote to Table II.

117  $\mu\text{l. CO}_2$  from 224  $\mu\text{l. pyruvate}$  in the first two-hour period, and 53  $\mu\text{l. CO}_2$  in the second two-hour period.

The results of a typical experiment with honeydew melon pulp are shown in Table IV. Here the rate of  $\text{CO}_2$  evolution from oxalacetate is almost twice as great before heat-inactivation as after heat-inactivation. This would be possible if the pyruvic carboxylase was sufficiently

active to decarboxylate the pyruvate as fast as it might be formed from oxalacetate by heat-stable catalyses. The pyruvic carboxylase is, in fact, considerably more active than in cucumber. In 120 minutes, the  $\text{CO}_2$  evolution has stopped at the theoretical level. Whether all of the heat-labile catalyses can be attributed to the pyruvic carboxylase is, of course, not certain, but, as in cucumber, there is no evidence for the presence of a true oxalacetic carboxylase.

### *Carboxylases in Seeds of the Leguminosae*

Green peas, green lima beans and soy beans were all found to contain active pyruvic carboxylase. The preparation from green peas decarboxylated 224  $\mu\text{l}$ . pyruvate completely in 20 minutes. The prepara-

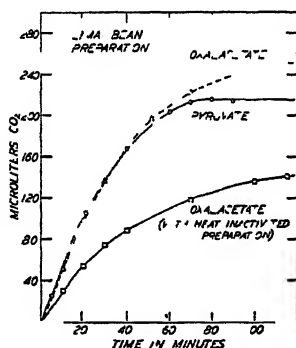


FIG. 5

The Action of a Lima Bean Preparation on Oxalacetate and Pyruvate  
Conditions as in Fig. 1.

tion from lima beans required 70 minutes to effect the same action. A soy bean suspension decarboxylated the same amount of pyruvate in 30 minutes. (The preparations are described in Table I.) Lima beans and soy beans were tested with oxalacetate before and after heat-inactivation and neither gave any clear cut evidence for the existence of an enzyme attacking oxalacetate directly. In Fig. 5 results obtained with green lima beans taken as a representative example are plotted. This preparation had been centrifuged and the experiment was done with the cloudy supernatant extract. Results obtained with soy beans are shown in Table IV. Although the  $\text{CO}_2$  evolution from oxalacetate

is decreased a little more than 50% after heat inactivation, the margin of difference is very small and cannot be regarded as significant in view of the presence of a very active pyruvic carboxylase.

### *Carboxylases in Cruciferae*

All common members of the mustard family (cabbage and related varieties, radish and turnip) that were tested contained pyruvic carboxylase. Results given in Figs. 6 and 7 and in Table IV refer to

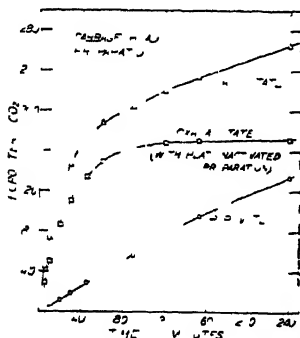


Fig. 6

The Action of a Cabbage Head Preparation on Ovalacetate and Pyruvate Conditions as in Fig. 1. Entire cabbage head used to make the preparation.

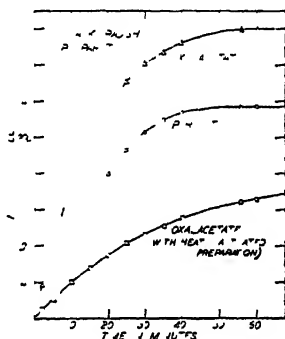


Fig. 7

The Action of a Black Radish Preparation on Ovalacetate and Pyruvate Conditions as in Fig. 1.

the preparations described in Table I. In addition to cabbage, Brussels sprouts, broccoli, white turnip, yellow turnip, horseradish, red radish and black radish, for which data are given in the Table and the Figures, cauliflower, kohlrabi and white radish were also tested. A single preparation from white radish showed low activity (301  $\mu$ l./hr.) but no further work was done with this source. Cauliflower decarboxylated pyruvate at the same rate as broccoli (Table IV). The enlarged stem of kohlrabi contained relatively more pyruvic enzyme, decarboxylation of 224  $\mu$ l. pyruvate being complete in 1.5 hours. Kohlrabi leaves, on the other hand, were inactive toward pyruvate. Since both stem and leaves were present in the cabbage head, tests were conducted to see whether, in this case, the distribution of the enzyme was similar to that in kohlrabi. Fig. 6 shows results with a cabbage head preparation made from both stem and leaves. The data

in Table IV obtained with preparations made from separated stem and leaves show that most of the pyruvic carboxylase activity is located in the stem.

The pyruvic carboxylase activity of some of the *Cruciferae* preparations decreased rapidly on standing. This was particularly true of the horseradish. An active preparation from this source was almost completely inactive toward pyruvate after standing overnight in the ice box. This inactivation could be partially prevented by dialysis against water. Since such dialysis also prevents some of the darkening of the extracts, presumably by removing the natural substrates of the oxidases, it seems probable that the action of the oxidases is associated with the inactivation of the pyruvic carboxylase. Precautions against such inactivation are necessary if the full enzyme activity of the original extract is to be measured. Finally, it should be noted that the rate of  $\text{CO}_2$  evolution from pyruvate, although it was always appreciable, varied from one preparation to another. Thus, the time required for complete decarboxylation of 224  $\mu\text{l}$ . pyruvate ranged from 1.75 to 3.5 hours for the white turnip, from 1.5 to 4 hours for the yellow turnip and from 1.7 to 3 hours for the red radish. The data listed are simply representative examples.

Examination of the data on *Cruciferae* to ascertain a possible direct action on oxalacetate by use of the criterion already described reveal several possible sources of oxalacetate carboxylase. In cabbage leaves, for example, the pyruvic enzyme is so weak that it cannot account for the extra  $\text{CO}_2$  evolution from the oxalacetate in the unheated sample, but the margin of difference is small and judgment of its significance should be deferred. However, cabbage stem, red radish and black radish all show definitely more than 50% decrease in rate of  $\text{CO}_2$  evolution from oxalacetate after heat inactivation of the enzyme preparation. In the first two instances, the results are not striking, but there can be no question of the evidence in the case of the black radish (Fig. 7). At 20 minutes a heat-inactivated preparation gave 70  $\mu\text{l}$ . carbon dioxide from oxalacetate, whereas an unheated preparation gave 232  $\mu\text{l}$ . 140  $\mu\text{l}$ . would be the maximum expected, unless the preparation contains a heat-labile fraction that attacks oxalacetate directly.

#### *Carboxylases in Umbelliferae*

Familiar members of the *Umbelliferae* include carrot, parsnip and parsley. Pyruvic carboxylase was found in the roots of all these species,

parsnip and parsley root being excellent sources of this enzyme, carrot showing somewhat lower activity (Table IV and Fig. 8). All these 3 sources gave evidence of the presence of a heat-labile factor acting directly on oxalacetate. In the case of the carrot the evidence was not strong, but in the case of parsnip it was as good as that with black radish. Of all the preparations tested, parsley root gave strongest indirect evidence for a heat-labile oxalacetic carboxylase. Fig. 8 shows that the rate of  $\text{CO}_2$  evolution from the  $\beta$ -keto acid was reduced to less than a quarter of the original by heat-inactivation.

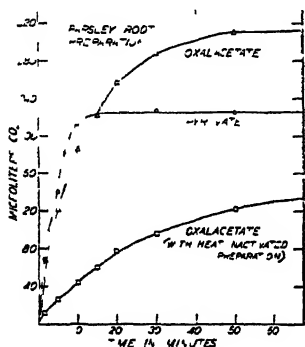


Fig. 8

The Action of a Parsley Root Preparation on Oxalacetate and Pyruvate Conditions as in Fig. 1.

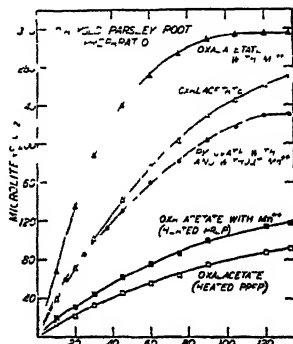


Fig. 9

The effect of manganese on the Carboxylase Action of a Dialyzed Parsley Root Preparation

Conditions as in Fig. 1. 0.1 ml. 0.01  $M$   $\text{MnCl}_2$  added as indicated to make final concentration of 0.0005  $M$   $\text{MnCl}_2$ .

### *The Separation of Oxalacetic Carboxylase from Pyruvic Carboxylase in Parsley Root Preparations*

Parsley root was chosen for further examination to test the validity of the indirect evidence employed to demonstrate oxalacetic carboxylase in the presence of pyruvic carboxylase. It was of particular interest to see whether the two activities could be separated and demonstrated independently.

That they were independent could be demonstrated by examination of a dialyzed preparation. Dialysis was carried out against 0.025  $M$  phosphate buffer, pH 7.4 for 48 hours at 4°C. The coagulum which formed during dialysis was centrifuged off

and the cloudy supernatant was tested for its action on oxalacetate and pyruvate with and without 0.0005 *M*  $\text{MnCl}_2$ . The results of the experiment are shown in Fig. 9. Manganese ions have no effect on the pyruvic carboxylase activity (which has been considerably diminished by dialysis). On the other hand, these ions cause an increase in the  $\text{CO}_2$  evolution from oxalacetate incubated with the unheated preparation and this increase is 7 times as great as the increase observed under the same conditions with the heat-inactivated preparation. These facts show that the heat-labile factor which attacks oxalacetate directly is non-dialyzable, that it is activated by manganese ions, and that it is independent of the pyruvic carboxylase activity since the latter was unaffected by manganese under the same conditions.

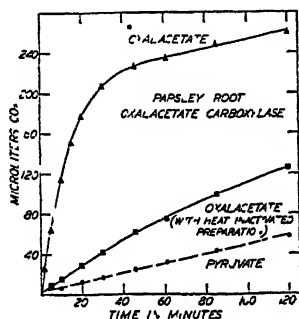


Fig. 10

#### Demonstration of Oxalacetic Carboxylase in Parsley Root

1 ml. clear enzyme preparation, 0.5 ml. 0.5 *M* acetate buffer, pH 5.0, 0.1 ml. 0.01 *M*  $\text{MnCl}_2$ , final volume made up to 2.0 ml. 224  $\mu\text{l}$ . oxalacetic acid or 224  $\mu\text{l}$ . sodium pyruvate tipped in at time 0.  $t = 30^\circ\text{C}$ .; gas phase, air.

Further inactivation of the pyruvic carboxylase was achieved by treating the dialyzed preparation with 1/20 volume 3 *M* acetate buffer, pH 5.0, allowing the solution to stand overnight in the ice box, centrifuging off the precipitate and dialyzing the supernatant against phosphate as before. Such a preparation, after another centrifugation, was usually perfectly clear, pale yellow in color and contained only a small amount of pyruvic carboxylase. The oxalacetic carboxylase activity could easily be demonstrated, however.

Fig. 10 shows the  $\text{CO}_2$  evolution from oxalacetate and from pyruvate observed in the presence of manganese. The presence of a heat-labile factor decarboxylating oxalacetate is apparent. When the  $\text{CO}_2$  evolved is equivalent to the oxalacetate added, the rate of  $\text{CO}_2$  evolution de-

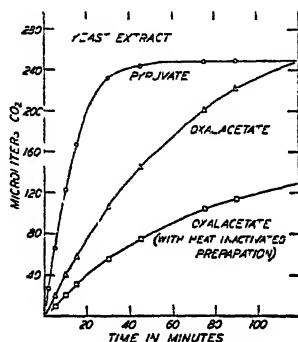


Fig. 11

#### The Action of a Lebedew Saft from Brewer's Yeast on Oxalacetate and Pyruvate Conditions as in Fig. 1.



creases to that observed with pyruvate. Analysis for pyruvate at this point shows the theoretical amount has been found. The results are analogous to those obtained with preparations of squash and pumpkin seeds, except that the latter show oxalacetic carboxylase activity without addition of divalent cations, whereas manganese is required by the parsley root enzyme. The activation by manganese of the preparation employed in the experiment of Fig. 10 was striking. Thus, in 20 minutes, the  $\text{CO}_2$  evolved from oxalacetate with unheated and heated preparations was 32  $\mu\text{l.}$  and 21  $\mu\text{l.}$ , respectively, in the absence of manganese, as compared with 177  $\mu\text{l.}$  and 29  $\mu\text{l.}$  in the presence of the divalent cation. The manganese ions only caused a 10% increase, however, in the rate of  $\text{CO}_2$  evolution from pyruvate.

These experiments are regarded as adequate evidence for the presence of an enzyme which acts directly on oxalacetate and is independent of pyruvic carboxylase. The results with parsley root also lend support to the criteria employed in the initial test system designed to demonstrate oxalacetic carboxylase in the presence of an active pyruvic carboxylase. Further work on the oxalacetic carboxylase of parsley root will be described in another publication.

#### *Action of Yeast Carboxylase on Oxalacetate*

For comparison with the results obtained with higher plants, crude yeast carboxylase preparations were subjected to the testing procedure devised for demonstration of direct action on oxalacetate. Fig. 11 shows the results obtained with a Lebedew juice from dried brewer's yeast. It can be seen that there is no conclusive evidence for the existence of a heat-labile factor acting directly on oxalacetate, since the rate of  $\text{CO}_2$  evolution with an unheated sample is never more than twice that observed with a heat-inactivated sample, although an active pyruvic carboxylase is present. Baker's yeast gave similar results except that the action of the extract on pyruvate was less, and the decrease observed in rate of  $\text{CO}_2$  evolution from oxalacetate after heating was correspondingly lower (Table IV).

Comparison of Figs. 8 and 11 shows clearly that the initial parsley root preparation has, relative to its pyruvic carboxylase activity, a greater heat-labile action on oxalacetate than does the preparation from brewer's yeast. The different relationships which obtain between the pyruvate and oxalacetate carboxylase activity in preparations from different sources (for example, parsley root and yeast) support

the conclusion that the "direct" heat-labile action on oxalacetate as detected by the test system, should not be attributed to the pyruvic carboxylase.

### *Oxidative Oxalacetic Carboxylase*

With none of the preparations tested was any evidence found for a pyruvic oxidase, *i.e.*, a factor which caused extra oxygen consumption in the presence of pyruvate. With oxalacetate, however, such extra oxygen consumption was sometimes observed. Effects were usually small. In the initial test system, cucumber and yellow squash pulp gave variable extra oxygen consumption of less than 20  $\mu$ l. in two hours.

A considerable number of more clear cut results were obtained with preparations tested after dialysis against water and the addition of  $\text{MnCl}_2$ . This procedure was adopted because a wide variety of animal tissue preparations have been found to oxidize oxalacetate under these circumstances (18). Spinach, Swiss chard and pineapple gave consistently positive results. Broccoli (inflorescence and stem), white turnip roots and kohlrabi (enlarged stem) gave smaller inconsistent effects which were never greater than 26  $\mu$ l. in two hours. The largest and most consistent effects were obtained with spinach. The dialyzed extract usually showed a high blank oxygen consumption. Thus, in one experiment, 1 ml. dialyzed extract consumed 43  $\mu$ l. oxygen and gave off 18  $\mu$ l.  $\text{CO}_2$ . When this extract was centrifuged, a bright green precipitate was separated from an inactive supernatant. Results obtained with the precipitate resuspended in water are given in Fig. 12. This particular preparation gave higher oxygen consumption and lower blanks than were usually obtained. It was not possible to repeat the quantitative results consistently, although all spinach preparations showed some evidence for the reaction. The oxygen consumption shown in Fig. 12 was not obtained in the absence of added manganous ions. The course of the gas exchange in this reaction is very similar to that observed with preparations from animal tissues. Thus, the oxygen consumption starts only after an initial lag period, and during the course of the oxidation two moles of  $\text{CO}_2$  are liberated for each mole of oxygen consumed. In pig heart, the product of such an oxidation has been identified as malonic acid and the active factor has been found to be metmyoglobin (18, 19). In spinach the active factor appears to be insoluble and its nature is not known.

*Summary of Negative Results*

The procedure chosen failed to give evidence for pyruvic carboxylase in preparations made from the following sources, which are not restricted to dicotyledons:

*Pyrus Malus* L.

Apple (Jonathon and Delicious)—mature fruits in which the bulk of the preparation came from the parenchyma

*Thuja occidentalis* L.

Arbor vitae—leaves and cones

*Asparagus officinalis* L.

Asparagus—young shoots

*Beta vulgaris* var. *crassa* Alef.

Beer—root

*Apium graveolens* L.

Celery—tuberous root

*Peucedanum graveolens* Benth. and Hook.

Dill—leaves

*Cichorium Endivia* L.

Endive—leaves

*Foeniculum officinale* All.

Fennel—seeds

*Linum usitatissimum* L.

Flax—seeds

*Taraxacum officinale* Weber.

Garden dandelion—leaves

*Allium Cepa* L.

Green onion—bulb

*Pisum sativum* L.

Green pea pods (seeds removed)

*Capsicum annuum* L.

Green pepper—whole fruit with seeds removed

*Cannabis sativa* L.

Hemp—seeds

*Brassica oleracea* var. *CaULO-Rapa* DC.

Kohlrabi—leaves

*Lactuca sativa* L.

Leaf lettuce—leaves

*Ananas sativus* Schult.

Pineapple—whole multiple fruit

*Solanum tuberosum* L.

Potato, white and red varieties—whole tuber with skin removed

*Spinacia oleracea* L.

Spinach—leaves

*Beta vulgaris* var. *Cicla* Moq.

Swiss chard—leaves

*Solanum Lycopersicum* L.

Tomato—whole fruit, seeds strained off

The inconclusive nature of the negative evidence must again be emphasized. The test was not devised to demonstrate small amounts of activity or easily inactivated enzymes.

In connection with the negative results obtained with potato tubers it should be pointed out that Bodnár in 1916 (20) reported pyruvic carboxylase activity in an alcohol-ether precipitate of a potato juice. We sometimes observed slight CO<sub>2</sub> evolution from pyruvate with potato juice but it was only of a magnitude of 5  $\mu$ l./hr. and it was regarded as negative. It is possible, however, that another testing procedure may demonstrate the presence of the enzyme in potato. Similar considerations may apply to the apple since the demonstration of alcohol formation in this fruit (11) makes the occurrence of pyruvic carboxylase seem likely.

The list of materials which failed to show positive results is useful,

however, because it indicates that in such cases other means than the test procedure described must be found for the purpose of demonstrating the enzyme.

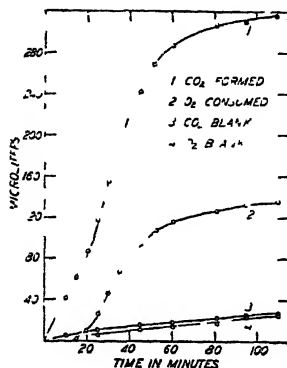


FIG. 12

The Oxidative Decarboxylation of Oxalacetate by a Spinach Preparation

1 ml. dialyzed and resuspended spinach in 0.05 *M* acetate pH 5.0, 0.0005 *M*  $MnCl_2$ , total volume 2.0 ml. 280  $\mu$ l. oxalacetic acid added at time 0. *t* = 30°C. Gas phase, air.

## DISCUSSION

A discussion of the role of oxalacetic carboxylases in plant metabolism should be deferred until more experimental evidence is available, particularly in regard to the reversibility of these enzymes. Meanwhile, certain differences between the known oxalacetic carboxylases may be pointed out. Thus, the crystalline seed globulins found to behave as oxalacetic carboxylases do not require added divalent cations after dialysis, whereas the parsley root enzyme, the pigeon liver enzyme and the bacterial enzymes seem to behave alike in requiring manganese or magnesium. The presence of divalent cations in the crystalline globulins has not, however, been excluded. The high catalytic activity of the heat-coagulated cucurbit seed globulins affords another point of contrast to the parsley root and pigeon liver enzymes. Finally, the cucurbit seed globulins are present in high concentration as storage proteins in the seeds (17), whereas evidence at hand indicates that the other oxalacetic carboxylases constitute a relatively low pro-

portion of the dry weight of the tissues in which they have been demonstrated.

One of the difficulties encountered in searching for oxalacetic carboxylase in plant tissues has been the wide distribution of pyruvic carboxylase. The high activity of some of the plant sources of pyruvic carboxylase described in this paper compares favorably with that of yeast. This is likewise true of the pyruvic carboxylase in cereal grains, according to Bartlett (7).

The question whether pyruvic carboxylase attacks oxalacetate directly or indirectly has been of particular concern in this investigation. It has been possible to demonstrate beyond question that several plant preparations which contain active pyruvic carboxylase must contain a heat-labile factor which attacks oxalacetate directly, but such a demonstration, in itself, does not indicate whether this factor is identical or not with the pyruvic enzyme. We have, however, on the basis of the combined data, reached the conclusion that, if any pyruvic carboxylase enzyme attacks oxalacetate directly, such a reaction must be so slow as to be negligible in comparison with the action of the enzyme on pyruvate itself. This conclusion assumes that pyruvic carboxylases prepared from different sources show similar specificities, and rests primarily on the fact that the action of several active pyruvic carboxylase preparations (notably yeast) on oxalacetate can be accounted for entirely as an indirect action. If others, of similar activity toward pyruvate, show clear-cut evidence for direct action on oxalacetate, such activity must be due to a factor separable from pyruvic carboxylase. Verification of this conclusion has been obtained for parsley root. Further examination of other preparations giving evidence for a heat-labile factor attacking oxalacetate directly is in progress. If the oxalacetic carboxylase in each of these preparations can be shown to be independent of pyruvic carboxylase, our conclusion will be confirmed.

The incidental observation that cucurbit seeds show a rapid increase in pyruvic carboxylase activity on soaking, raises interesting questions in regard to the physiological significance of pyruvic carboxylase during germination. Goddard's (21) conclusion that pyruvic carboxylase plays a role in controlling respiration of germinating ascospores of *Neurospora* suggests the existence of related phenomena in an entirely different plant group.

## ACKNOWLEDGMENTS

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## SUMMARY

1. The crystalline globulins of squash and pumpkin seeds have been found to display oxalacetate carboxylase activity.

2. The presence of pyruvic carboxylase has been demonstrated directly in cucurbit seeds and pulp, in the seeds of several legumes not previously examined, and in all the common members of the *Cruciferae* (cabbage, radish, turnip) and the *Umbelliferae* (carrot, parsley, parsnip) examined.

3. The action of these pyruvic carboxylase preparations on oxalacetate has been examined and evidence obtained that several, especially parsley root, parsnip and black radish, possess a heat-labile factor which can attack oxalacetate directly. In the case of parsley root, which was subjected to further investigation, oxalacetic carboxylase was shown to be independent of pyruvic carboxylase.

4. An oxidative decarboxylation of oxalacetate has been demonstrated in dialyzed spinach preparations supplemented with  $MnCl_2$ .

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# The Enzymatic Desamination, Dephosphorylation and Degradation of Nucleic Acids to Dialyzable Substances

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## INTRODUCTION

In earlier reports (1, 2) a study was made at neutral pH of the desamination of ribosenucleic (yeast) and of desoxyribosenucleic (thymus) acids added to aqueous extracts of rat spleen, and the following phenomena were noted: (1) the rate of appearance of ammonia is progressive with time, reaching a maximum value of 100  $\gamma$  of ammonia nitrogen evolved from 5 mg. of either type of nucleate; (2) although in dialyzed, salt-free extracts of spleen, ribosenucleic acid is desaminated to the same extent as in fresh extracts, desoxyribosenucleic acid is not desaminated at all; (3) the capacity to desaminate the desoxyribosenucleate can, however, be completely restored to the dialyzed extract by adding at 0.01–0.001 *M* concentration the sulfate, acetate, nitrate or halide (except fluoride) of any one of the alkali or alkaline earth metals (except beryllium), the hydrochlorides of such organic bases as guanidine or arginine, the chlorides of manganese, cobalt and nickel, and the neutral sodium salt of glutamic acid; (4) the sodium salts of fluoride, bicarbonate and phosphate not only fail to restore the capacity to desaminate desoxyribosenucleate to the dialyzed extract but are also inhibitory when added to the fresh extracts; and (5) when dialyzed extracts of spleen are digested with ribosenucleate much more phosphorus appears in dialyzable form than when fresh extracts of this tissue are used. These findings have since been repeatedly confirmed.

When the digests of nucleate and originally fresh spleen tissue extract were heated at 100°C. at the end of the incubation period and then dialyzed, no appreciable



organic nitrogen or phosphorus was noted in the dialyzate and on the basis of this observation it was believed that the desamination of the nucleates occurred when they were highly polymerized molecules (1, 2).

Repetition of these experiments recently yielded results in accord with those obtained earlier, but this time, carrying the observations further, we noted considerable amounts of excess pentose in dialyzates from the heated digests. It therefore seemed probable that the nucleates are actually split into smaller, dialyzable fragments during the digestion period. We have subsequently established this as a fact by incubating mixtures of *fresh* tissue extracts with added nucleates in cellophane bags immersed in saline solutions, and noting invariably a progressive increase in the amount of dialyzable organic nitrogen and phosphorus, pentose, and material absorbing light with a maximum at 2600 Å (see below).

The failure to note appreciable amounts of organic nitrogen and phosphorus in dialyzates of the *heated* digests is very probably attributable to the adsorption and combination of such nucleate fragments on the surface of the tissue proteins denatured by heat in their presence. That there is a distinct reaction between protein and nucleic acid in aqueous solution at elevated temperatures has been pointed out (1, 2, 3). Some such explanation may also be applied to the results obtained by Schmidt and Levene (4) who noted no dialyzable organic phosphorus in digests of ribosenucleate with heated pancreatin, and, on the basis of our own experience, we are in substantial agreement with the interpretation which Loring (5) placed on these results. It is evidently necessary to modify our earlier viewpoint to the extent that the ammonia nitrogen noted in digests of nucleates with tissue extracts may have been derived not only from the intact nucleate but from any of its intermediate degradation products down to the free purine, and that the designation of nucleodesaminase, previously applied only to the intact nucleate, may for present purposes be an inclusive term referring to one or more systems responsible for enzymatic desamination of these products.

The present communication consists of further studies of the enzymatic degradation of the nucleic acids, including desamination, dephosphorylation, and the formation of dialyzable products. Studies of the desamination and dephosphorylation of the individual nucleotides, *etc.*, as well as studies on tumors are described elsewhere (6, 7).

## EXPERIMENTAL RESULTS

### *The Desamination and Dephosphorylation of Nucleates and Nucleotide Mixtures in Fresh and Dialyzed Rat Tissue Extracts*

The amount of ammonia and inorganic phosphate split from nucleates added to fresh and dialyzed extracts of various rat tissues

in the presence of different salts is noted in Table I. It should be emphasized again that the amounts of ammonia and phosphate so noted represent the overall appearance of these products during the

TABLE I

*Effects of Salts on the Desamination and Dephosphorylation of Nucleates and Nucleotide Mixtures in Fresh and Dialyzed Rat Tissue Extracts<sup>1</sup>*

Substrate <sup>2</sup>	Salt	Liver				Kidney				Spleen			
		Fresh		Dialyzed		Fresh		Dialyzed		Fresh		Dialyzed	
		N	P	N	P	N	P	N	P	N	P	N	P
Ribosenucleate	None	23	50	56	124	86	240	92	228	92	20	96	65
	MgSO <sub>4</sub>	26	50	60	130	84	236	92	230	92	20	96	65
	NaHCO <sub>3</sub>	7	40	10	14	74	250	88	240	43	10	14	18
	NaF	7	32	0	10	62	248	40	140	28	10	0	8
Mixture of four ribose nucleotides <sup>4</sup>	None	44	106	44	106	94	300	94	300	90	52	90	52
Desoxy ribosenucleate	None	5	40	0	0	58	180	0	0	80	6	5	4
	NaCl	6	40	20	110	58	200	103	210	80	6	88	160
	KCl	6	40	20	112	58	210	102	200	80	6	88	150
	CaCl <sub>2</sub>	6	42	22	124	58	208	102	220	50	6	84	180
	MgCl <sub>2</sub>	6	50	24	156	58	220	104	220	84	8	90	210
	Arginine HCl	6	45	20	120	58	210	100	195	56	6	92	200
	NaHCO <sub>3</sub>	2	35	0	8	20	200	0	0	10	3	0	0
	NaF	0	0	0	0	15	210	12	0	0	2	0	4

<sup>1</sup> Digests consisted of 1 cc. aqueous tissue extract (equivalent to 166 mg. tissue) plus 1 cc. substrate. Extracts (and nucleate solutions) were dialyzed for 24 hrs. against distilled water at 5°C. Salts added as 0.2 cc. of 0.15 *N* stock solution to extracts prior to mixing with substrate. Incubation period was 5 hrs. at 37°C. Results are given in terms of increase in  $\gamma$  of ammonia N and inorganic phosphate P over controls in which 1 cc. of water was used in place of substrate. Ammonia determinations made as before (2). Phosphorus determinations by method of Fiske-Subbarow; identical results obtained by the method of Lowry and Lopez (3). pH of the digests was 6.4–6.8; in the presence of bicarbonate the pH was 7.6. Control experiments on digests brought to pH 7.6 with dilute NaOH gave results identical with those at pH 6.4–6.8.

<sup>2</sup> Substrates at 0.5% stock solution in water. Substrate solutions used with dialyzed extracts were themselves dialyzed prior to use. Sodium yeast nucleate used as model for ribosenucleate, sodium thymus nucleate (Hammarsten) as model for desoxy-ribosenucleate. Total P in former was 8.0%, in latter was 8.5%.

<sup>3</sup> In final concentration of 0.014 *N* in digests. Effective range found to be 0.001–0.10 *N*.

<sup>4</sup> Each cc. of the ribose nucleotide equimolar mixture contained 1.24 mg. adenylic acid, 1.28 guanylic acid, 1.20 mg. cytidylic acid and 1.20 mg. uridylic acid, the total being equivalent, on a tetranucleotide basis, to the 5 mg. of ribosenucleate employed. The nucleotide mixture solution was brought to pH 6.8 by addition of dilute NaOH. Description of the nucleotides and of the nucleates is given elsewhere (6).

digestion period and may have been derived from any or all stages of the breakdown of the nucleates into smaller fragments.

The following points in Table I may be noted: (1) Under the conditions employed, tissue extracts require salts for the metabolism of desoxyribosenucleate but not for that of ribosenucleate; (2) except in extracts of kidney, sodium bicarbonate and sodium fluoride have an inhibiting effect on the desamination and dephosphorylation of the nucleates whether in fresh or in dialyzed extracts; (3) dialyzed extracts of liver and spleen produce more dephosphorylation of ribosenucleate than do fresh extracts, while dialyzed extracts of liver produce more de-amination of this substrate; (4) dialyzed extracts of kidney plus effective salts produce more desamination of desoxyribosenucleate but no more dephosphorylation than do fresh extracts; (5) dialyzed extracts of all the tissues have little or no effect in desaminating or dephosphorylating desoxyribosenucleate; (6) addition of the chlorides of sodium, potassium, calcium, magnesium or arginine considerably enhances, beyond that of the fresh tissue, the capacity of the dialyzed extracts of liver to desaminate and dephosphorylate desoxyribose-nucleate and of spleen to dephosphorylate this substrate; (7) addition of bicarbonate or fluoride to the dialyzed extracts of the tissues fails to restore either desamination or dephosphorylation capacity for desoxyribosenucleate; (8) all the effective chlorides appear to be nearly equally effective in the degree to which each restores or enhances, beyond that of the fresh tissues, the desamination and dephosphorylation capacity of the dialyzed tissues; and (9) although in the fresh tissue extract, the dephosphorylation of ribosenucleate exceeds that of desoxyribosenucleate, the reverse is true in dialyzed extracts of liver and of spleen.

These findings are generally consistent with those noted earlier (1, 2) and, by extension of the investigations to tissues other than spleen, have emphasized the phenomena. The considerably greater amount of ammonia and inorganic phosphate noted in mixtures of nucleate with dialyzed tissues and added salt, as compared with similar mixtures with fresh tissues, is particularly noteworthy in view of the fact that the desamination and dephosphorylation of individual ribose nucleotides occur to the same extent in fresh and dialyzed tissue extracts (6).

As noted in Table I, the amount of ammonia and of phosphate which appears in digests of ribosenucleate in dialyzed tissue extracts is very nearly the same as in digests of ribose nucleotide mixtures equivalent

in amount to the ribosenucleate. From this it would appear as if ribosenucleate digested in dialyzed tissue extracts behaved as if it were an equivalent mixture of four equimolar nucleotides. The essential difference in the amount of ammonia and phosphate split from ribosenucleate on the one hand, and from the ribose nucleotide mixture on the other hand, is revealed in fresh extracts of the tissues.

The requirement of the enzymes in the tissue extracts for the presence of salt in order to desaminate and dephosphorylate desoxyribosenucleate, as contrasted with the apparent dispensability of salts in the desamination and dephosphorylation of ribosenucleate, can apparently be satisfied to an equal extent by a wide variety of monovalent and divalent salts. Experiments on the time course of the desamination and dephosphorylation of desoxyribosenucleate by dialyzed rat spleen extract showed that the rate of reaction was very nearly the same in the presence of 0.01 *M* NaCl, MgCl<sub>2</sub> or arginine HCl. The restorative capacity of salts is not confined to those encountered physiologically, as shown in Table II.

It is evident from the data in Table II (as well as Table I) that the restoration and inhibition of desamination and dephosphorylation run parallel. The wide variety and parallel effect of the active salts suggests that they act upon a state of the nucleate prior to the enzymatic desamination and dephosphorylation, unfolding and splitting the nucleate by a relatively non-specific, non-enzymatic depolymerization to a size, or series of sizes, susceptible to nuclease activity concurrent with or followed by desamination and dephosphorylation. That salts can effect changes in size and shape of desoxyribosenucleate has been demonstrated (*cf.* 9). The fact that dephosphorylation follows a preliminary degradation of the polymerized nucleate has been demonstrated by Schmidt (10), Schmidt, Pickels and Levene (11), Schmidt and Thannhauser (12) and Laskowski and Seidel (13). In addition to the relatively non-specific effect of salts on the state of aggregation of the nucleates, there is also a specific effect of certain ions, such as fluoride or bicarbonate, on the enzymatic splitting of ammonia and phosphate from the nucleate digests (Table I).

Studies of acid-soluble phosphorus and inorganic phosphate phosphorus in digests of desoxyribosenucleate with fresh and dialyzed rat spleen extract (Table III) revealed the following: (1) Bicarbonate added to the fresh extract inhibits the formation of acid-soluble phosphorus; (2) in the dialyzed tissue very little acid-soluble phos-

TABLE II

*Effect of Salts on Restoration of Capacity to Produce Ammonia and Inorganic Phosphate from Desoxyribosenucleate in Dialyzed Extracts of Rat Spleen<sup>1</sup>*

Salt	Ammonia N γ	Phosphate P γ
None	0 <sup>2</sup>	0 <sup>3</sup>
Sodium fluoride	0	0
Sodium chloride	90	150
Sodium bromide	92	158
Sodium iodide	90	145
Sodium nitrate	90	160
Sodium sulfate	88	160
Sodium citrate	90	120
Sodium succinate	88	140
Sodium glutamate	89	200
Sodium acetate	86	180
Sodium nitroprusside	90	180
Sodium bicarbonate	0	0
Lithium chloride	92	190
Potassium chloride	90	185
Rubidium chloride	94	180
Cesium chloride	90	172
Beryllium sulfate	0	0
Magnesium chloride	90	210
Calcium chloride	94	200
Strontium chloride	92	190
Barium chloride	90	190
Manganese chloride	88	200
Nickel chloride	80	180
Cobalt chloride	82	180
Guanidine hydrochloride	94	190
Arginine hydrochloride	92	190

<sup>1</sup> Digests and experimental conditions as in Table I.

<sup>2</sup> Value for fresh extract 84 γ ammonia N.

<sup>3</sup> Value for fresh extract 6 γ inorganic phosphate P.

phorus is present, but on addition of the chlorides of sodium, magnesium or arginine a very considerable amount, in excess of that revealed in fresh tissue, is formed; (3) bicarbonate shows no such restorative effect in dialyzed extract; and (4) in the dialyzed, salt-containing extract relatively little organically-bound, acid-soluble phosphorus is present, by far the greater proportion of the total acid-soluble phosphorus being inorganic phosphate.

Comparison of the last two columns in Table III suggests that the organically-bound, acid soluble phosphorus derived from the nucleates in the fresh digests is, in the dialyzed, salt-containing extract, further split to inorganic phosphate. Thus the considerable rise in the amount of inorganic phosphate in digests of desoxyribosenucleate in dialyzed extracts and salts as compared with fresh extracts, is caused to a large extent by the splitting to inorganic phosphate of the acid-soluble, organically-bound phosphorus produced from the nucleate.

It is evident that in the course of dialysis something is removed, the absence of which from the tissue extracts permits the appearance of more ammonia and inorganic phosphate from nucleate digests than

TABLE III  
*Acid-Soluble Phosphorus in Digests of Desoxyribosenucleate in Fresh and Dialyzed Rat Spleen Extracts<sup>1</sup>*

Salt	Total Acid-soluble P		Organic Acid-soluble P <sup>2</sup>	
	Fresh	Dialyzed	Fresh	Dialyzed
None	76	8	70	4
NaCl	100	170	94	10
MgCl <sub>2</sub>	100	214	92	4
Arginine HCl	94	210	88	10
NaHCO <sub>3</sub>	15	0	12	0

<sup>1</sup> Digests and experimental conditions as in Table I. At end of incubation period, 1 cc. of 5% fresh trichloroacetic acid was added to each tube, the mixture centrifuged and total P determined in supernatant. Results given in terms of  $\gamma$ .

<sup>2</sup> Total acid-soluble P minus inorganic phosphate P from Table I.

is evident in the fresh extract. Two possible explanations for this may be based (1) on the assumption that there is present in the fresh extract of liver or spleen some inhibitor for desamination and dephosphorylation which is removed by dialysis, or (2) on the assumption that there may be some acceptor for phosphate and ammonia in these tissues which is likewise lost on dialysis. According to the latter explanation, the nucleic acids or their larger split products but not the simple nucleotides may function as phosphate and ammonia donors—since the metabolism of the nucleotides is not affected by dialysis (*vide infra*). The kidney in either case must evidently lack either inhibitor or acceptor.

Fresh extracts of the various tissues show very decided differences among themselves in the amounts of ammonia and phosphate formed in the presence of nucleates, kidney being by far the most active. Since in the dialyzed extracts of liver and spleen, the amount of phosphate observed tends to increase and approach the value noted in the kidney extracts, it would appear that the differences between such tissues as liver, spleen and kidney tend to diminish when the extracts of such tissues are dialyzed and then treated with effective salts. It might appear that to some extent the differences in nucleic acid metabolism noted in fresh extracts of various tissues are due to dialyzable components in these tissues. The residual differences between the tissues observed in the dialyzed extracts are to be ascribed to differences in the activity of the component enzyme systems.

If all of the phosphorus in the nucleates were hydrolyzable phosphate, the theoretical amount for the amount of substrates used would be 400–450  $\gamma$  (Table I). On the basis of a tetranucleotide structure, the nucleates should possess 3 amino groups per tetranucleotide unit or for the amount of substrates used, 150  $\gamma$  of amino nitrogen. The data in Table I reveal that the amounts of nitrogen and phosphorus fall considerably short of the theoretical. Studies on the time course of the reaction (6) showed that the maximum amount of ammonia nitrogen found for all tissues, with one exception, was 100  $\gamma$ . The sole exception was in the case of mouse kidney extracts which, when incubated with either ribosenucleate or desoxyribosenucleate, yielded 150  $\gamma$  of ammonia nitrogen from 5 mg. of substrate. Every other tissue studied could desaminate adenylic and guanylic acids but not cytidylic acid, whereas extracts of mouse kidney are unique among those mammalian tissues studied in being able to desaminate all three (6).

#### *The Degradation of the Nucleates into Dialyzable Components*

To avoid coagulation of extract proteins prior to the determination of dialyzable components in nucleate digests, incubation and dialysis were carried on simultaneously. This was accomplished by adding tissue extracts and nucleates to small cellophane bags immersed in water or salt solutions of varying composition and incubating at 37°C. The conditions employed in experiments from which the data in Table IV are derived were the following: Fifty cc. tubes containing 20 cc. of .01 M NaCl into which had been inserted a small cellophane bag were employed. To the cellophane bags were added 4 cc. of a rat liver extract

obtained by grinding the tissue with sand and extracting with 3 volumes of water, and 2 cc. of a 0.5% solution of ribonucleic or desoxyribonucleic acid. Blanks contained water in place of nucleates. Following a 5 hour incubation period the cellophane bags were withdrawn and discarded and analysis made upon the dialyzate. Calculations were based on the total volume of the system and expressed as increments over blanks. The 5 hour incubation period was sufficient to give maximal values for dialyzable substances.

The application of this method to the study of purified nucleases and various tissue extracts is described elsewhere (7). Varying concentrations of salt, extract and nucleates have been found to influence profoundly the degradation of nucleic acids into dialyzable components. Data on digests of the nucleates with rat liver extracts are given in Table IV.

TABLE IV  
*Dialyzable Products from Digests of Nucleates and Rat Liver Extracts*

Expt	Nucleate <sup>1</sup>	Dialyzable Products <sup>2</sup>				
		Total N	Ammonia N	Total P	Ribose <sup>3</sup>	Desoxy- ribose <sup>4</sup>
		mg.	mg.	mg.	mg.	mg.
1	Ribonucleic Acid	1.14	.10	.73	2.4	—
	Desoxyribonucleic Acid	1.14	.12	.65	—	2.6
2	Ribonucleic Acid	1.25	.12	.92	2.82	—
	Desoxyribonucleic Acid	1.17	.12	.92	—	2.15
3	Ribonucleic Acid	1.12	.10	.60	2.6	—
	Desoxyribonucleic Acid	1.12	.10	.59	—	1.9

<sup>1</sup> 10 mg. of nucleate used for each digest.

<sup>2</sup> On basis of 10 mg. nucleate: total N = 1.5 mg.;  $\text{NH}_3$  N = .300 mg.; total P = .9 mg.; ribose and desoxyribose 4.0–4.6 mg.

<sup>3</sup> Determined by an adaptation of the furfural method described by Davidson and Waymouth (14). Values obtained here are half those expected in theory. This is interpreted as a failure of hydrolysis of the pyrimidine linkage under the conditions employed.

<sup>4</sup> Determined by the diphenylamine reaction. Therefore, pyrimidine nucleotides do not react and values for desoxyribose in desoxyribonucleic acid will be approximately half the amount that should appear in theory.



Data consistent with those reported in Table IV were obtained by removing an aliquot volume from the dialyzate and analyzing in the Beckmann ultraviolet spectrophotometer for the relative height of the absorption curve at 2600 Å (7). The known height of the absorption curve for the nucleates was used for comparison as well as the absorbing material evolved from the tissue extract controls. No more than 5% of the ribosenucleate or desoxyribosenucleate in aqueous solution was spontaneously dialyzable.

The conditions under which the dialytic experiments described in Table IV were performed were of course somewhat different from those described in Tables I-III, inasmuch as, in the former case, the products of the reaction dialyzed off as fast as they were formed or were degraded to particles of sufficiently small size. Under such dialytic conditions, 80% of the substrate is dialyzable at the end of the 5 hour incubation period, while some 40% of the theoretical ammonia, 70-100% of the total phosphorus, and about 50% of the theoretical sugar, appear. There is, therefore, little question that nearly all of the nucleate molecules studied under these conditions are degraded into particles sufficiently small enough to pass through a cellophane membrane. Preliminary studies on the simultaneous estimation of rates of desamination, dephosphorylation and degradation into dialyzable components suggested that the rates of all three processes were very much the same. Which reaction occurs first cannot be determined exactly at the present time, but it would appear that certain of these reactions must occur with great rapidity.

Studies (7) on the purified desoxyribosenuclease of McCarty (15) have revealed that this enzyme preparation splits desoxyribosenucleate into dialyzable particles. To effectuate this, the presence of any one of a number of neutral monovalent and divalent salts has been found to be essential (7). Thus, the splitting of desoxyribosenucleate into smaller particles is facilitated by sodium, magnesium or calcium salts. However, similar to the results of McCarty, the alteration in viscosity of solutions of the nucleate is dependent on the presence of magnesium (or manganese) salts and does not occur in the presence of calcium salts. Full details of these studies will be given elsewhere (7).

#### SUMMARY

The amount of ammonia and inorganic phosphate which appears in digests of ribosenucleate, of desoxyribosenucleate and of an equiv-

alent and equimolar mixture of the four ribosenucleotides has been estimated in fresh and dialyzed extracts of normal rat liver, kidney, and spleen.

Under similar experimental conditions, the amount of phosphate which appears in digests of ribosenucleate with dialyzed extracts of liver and spleen, and the amount of ammonia in digests with dialyzed liver, are much greater than with fresh extracts of the same tissues. The amounts of phosphate and of ammonia split from the ribosenucleotide mixture are the same in fresh and in dialyzed extracts, are greater than those noted in digests of ribosenucleate in fresh extracts and are very nearly equal to those noted in digests of ribosenucleate in dialyzed extracts. Ribosenucleate thus appears to possess a susceptibility to desamination and dephosphorylation in dialyzed extracts very similar to that of an equivalent mixture of adenylic, guanylic, cytidylic and uridylic acids in equimolar proportions.

In the absence of added salt in dialyzed extracts, desoxyribosenucleate is neither desaminated nor dephosphorylated. In the presence of 0.01 *M* salts of various type in dialyzed extracts, the dephosphorylation and desamination of desoxyribosenucleate exceed that shown in corresponding fresh extracts of the same tissues. This activation does not occur if the added salt is either fluoride or bicarbonate.

Under the same conditions, fresh extracts of kidney desaminate and dephosphorylate the nucleates to a nearly maximum extent, fresh extracts of spleen desaminate considerably but dephosphorylate very little, while fresh extracts of liver desaminate and dephosphorylate relatively little. In the dialyzed extracts of these tissues, the differences among them tend to diminish as both desamination and dephosphorylation of the nucleates rise above the values noted in the weaker tissues.

In digests of desoxyribosenucleate in fresh extracts of spleen, the greater part of the acid-soluble phosphorus is organically-bound, whereas in digests in dialyzed extracts the greater part of the acid-soluble phosphorus is inorganic phosphate.

The possibility that the nucleates, as contrasted with the nucleotides, serve as phosphate donors, is considered.

Dialyzable split products of the digests of the nucleates with fresh, salt-treated extracts of rat liver were noted and the relation of the sequence of degradation of the nucleates to the appearance of ammonia and inorganic phosphate is briefly considered.

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# Mutant Strains of *Neurospora* Requiring Nicotinamide or Related Compounds for Growth\*

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## INTRODUCTION

The biological importance of nicotinic acid and nicotinamide has been appreciated for many years. One is known to be a constituent of coenzymes, either may function as a growth factor for bacteria, and both serve as pellagra-preventive factors for man (see review 5). Despite their importance, little information is available regarding their biological synthesis. Numerous related compounds have been tested for nicotinic acid activity in various organisms (5) but, in general, only those which are readily hydrolyzable to nicotinic acid are active. Though recent work indicates the importance of tryptophan to nicotinic acid synthesis in rats (11), in no instance has a compound been found which could definitely be regarded as an immediate intermediate in the synthesis of nicotinic acid or nicotinamide.

Several strains of *Neurospora crassa* have been isolated which are genetically blocked in the biosynthesis of nicotinic acid and consequently require this substance for growth. Investigation of these strains has shown the production of a normal precursor of nicotinic acid by one of them. The present paper deals with a general study of these mutant strains and with the isolation of this nicotinic acid precursor.

## EXPERIMENTAL METHODS

Quantitative estimation of the growth response of mutant strains was made by culturing them for 72 hours at 25°C. in 125 ml. Erlenmeyer flasks containing 20 ml.

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of medium. After incubation the mycelial pads were removed, dried at 100°C. and weighed.

The basal medium used throughout is that described elsewhere (3, 7).

Additions to the basal medium were usually made prior to autoclaving. In all tests involving the precursor, however, solutions to be tested were sterilized by passage through a bacteriological sintered glass filter, and aliquots of this were then added to sterile basal medium.

Stock cultures of the strains listed in Table I are maintained either on the basal medium supplemented with 1 mg./l. of nicotinamide and solidified with 1.5% agar, or on the yeast extract medium described elsewhere (3).

### STRAINS

Following treatment with X-rays or ultraviolet radiation, 5 strains of *Neurospora crassa* have been obtained which require nicotinic acid or nicotinamide for growth (3). These 5 strains, with their culture characteristics, are listed in Table I.

TABLE I

*Mutant Strains of Neurospora Requiring Nicotinic Acid or Nicotinamide for Growth*

Strain Number	Treatment	Nicotinamide Required to Give Maximum Growth in 72 Hours at 25°C. $\gamma$ /ml.	Pigment Production in Medium
3416	X-ray	1.0	—
4540	X-ray	1.0	+
39301	Ultraviolet	1.0	+
39401	Ultraviolet	1.0	—
43002	Ultraviolet	1.0	+

All 5 strains have nearly the same growth requirement for nicotinamide. A typical growth curve is shown in Fig. 1. Nicotinic acid, tested in basal medium without addition of buffer, is about one-fourth as active as nicotinamide for all strains (Fig. 1). In contrast to that of nicotinamide, the activity of nicotinic acid is dependent upon the pH of the medium. As shown in Table II, the acid is inactive when tested in buffered medium at pH 6.6 but is as active as nicotinamide in a medium buffered at pH 4.6. At pH 6.6 nicotinic acid is 97% dissociated; at pH 5.6, 75%; and at pH 4.6, only 22% dissociated. The close correlation between activity of nicotinic acid and dissociation values suggests that only the undissociated acid is active. Similar observations have been made with nicotinic acid requiring mutant strains of

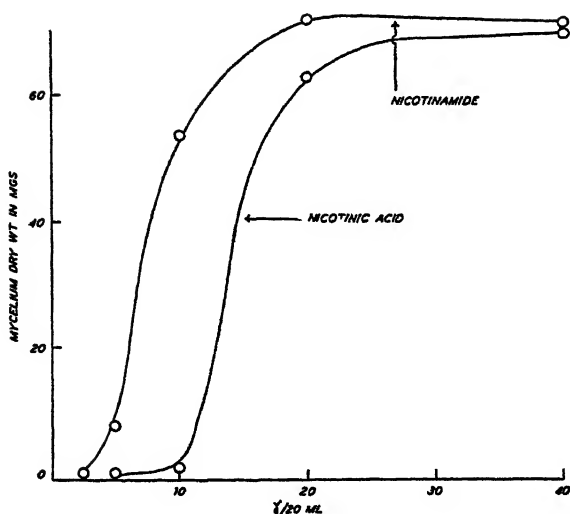


FIG. 1

Typical Growth Curve of a *Neurospora* Mutant Requiring Nicotinamide or Related Compounds for Growth

*Escherichia coli* (10). The primary effect of pH is probably on entrance into the cell, since the internal pH would not be expected to be changed greatly by alteration of the external pH from 6.5 to 4.5, suggesting that only the undissociated acid is taken up. After correction

TABLE II

Activity of Nicotinic Acid on the Growth of Strain 4540 Cultured in Medium Buffered at Various pH Values with 0.08 M McIlwaine's Standard Buffer

Nicotinic Acid Added γ/20 ml.	Weight of Mycelium Cultured 72 Hours at 25°C. (mgs.) at pH		
	4.6	5.6	6.6
0	0	0	0
4	15	0	0
8	53	0	0
12	57	0	0
16	64	3	0
20	60	8	0
24	60	59	0
28	63	65	0

ence in activity between these two compounds for any of the mutants. for dissociation of nicotinic acid is made, there is no significant differ-

As noted in Table I, 3 of the mutant strains are characterized by production of a pigment which accumulates in the medium. After several days' growth on either solid or liquid medium a reddish-brown pigment appears and accumulates in the medium. The relation of this pigment to nicotinic acid precursors will be considered further in a later section.

### GENETIC DATA

Beadle and Coonradt (2) have described a simple method of detecting genetic differences between mutant strains based on the use of heterocaryons. Heterocaryon tests were made in all possible combinations between the five strains listed in Table I. The tests were carried out by inoculating flasks of basal medium with each of two different strains. These were cultured for 5 days at 25°C. and the mycelial pads dried and weighed. The results are shown in Table III. Lack of hetero-

TABLE III

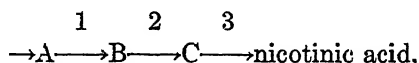
*Heterocaryon Formation between Mutant Strains Requiring Nicotinic Acid for Growth*  
Growth as milligrams mycelium dry weight.

	43002	39401	39301	4540	3416
3416	0.0	46.5	48.4	34.4	0.0
4540	0.0	32.5	0.0	0.0	—
39301	0.0	58.0	0.0	—	—
39401	8.2	0.0	—	—	—
43002	0.0	—	—	—	—

caryon formation does not necessarily constitute evidence of genetic identity. Growth as a result of heterocaryon formation, however, is a strong indication of genetic difference. Heterocaryon tests clearly indicate three genetic classes: I, 3416; II, 4540 and 39301; III, 39401. The relation of strain 43002 to the others is not entirely clear. Inter-crosses among strains 3416, 4540 and 39401 confirm the conclusion that they are genetically distinct. The first two are known to show sex linkage, while the third is differentiated from normal by a gene that is not sexlinked. It is evident, therefore, that among the 5 strains requiring nicotinic acid, there are 3 distinct types, each differing from the parent strain by a single gene.

*Activity of Known Compounds*

The fact that alteration of any one of three different genes results in a requirement for nicotinic acid suggests that there are at least three separate reactions involved in nicotinic acid synthesis (1, 4). This sequence might be represented as



with genes 1, 2 and 3 controlling the consecutive steps. Several known compounds have been tested on the 3 genetic types in the hope that a known compound might be found active for one genotype, but inactive for another. If such a compound were found it might represent one of the postulated precursors. The following compounds were tested:

Pyridine	<i>dl</i> -Ornithine
Piperidine	<i>l</i> -Proline
Piperidine-3-carboxylic acid	$\alpha$ -Amino- <i>n</i> -valeric acid
$\beta$ - and $\gamma$ -Picoline	$\alpha$ -Amino- <i>n</i> -caproic acid
Trigonelline*	<i>l</i> (-)-Tryptophan (tested in a mixture of amino acids)

None of these compounds is active for any strain and it, therefore, seems unlikely that synthesis involves either carboxylation of pyridine or dehydrogenation of piperidine.

*Natural Precursors*

In the scheme of synthesis of nicotinic acid shown above, precursors A, B and C should each be accumulated by one of the three strains provided these substances are stable and not further metabolized by the organism. Substance C should be accumulated by the strain blocked at 3 and should possess activity for strains 1 and 2. Similarly, the strain blocked at point 2 should accumulate substance B, which should serve as a growth factor for strain 1.

As a test for the accumulation of C or B, cross feeding experiments were carried out. The three genetically different strains, 3416, 4540 and 39401 were cultured on a shaking machine in basal medium supplemented with sufficient nicotinamide to give approximately three-quarters maximum growth (50  $\gamma$ /100 ml.). After incubation for

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\* The authors are indebted to Merck & Co., Rahway, N. J., for a sample of this compound.



72 hours at 25°C. the medium was filtered free of mycelium and aliquots of each culture filtrate added to basal medium for testing in the usual manner. After sterilization, the flasks were inoculated with the three strains.

The results of a typical experiment are shown in Table IV. Fig. 2 shows the activity of filter-sterilized 4540 culture filtrates on growth of strain 39401. From these data it is evident that strain 4540 produces a substance with nicotinic acid activity for strain 39401. This substance

TABLE IV

*Cross-Feeding Experiment between Mutant Strains*

+ indicates growth. - indicates no growth.

Inoculum Strain	Culture Filtrate from Strain		
	3416	4540	39401
3416	-	-	-
4540	-	-	-
39401	-	+	-

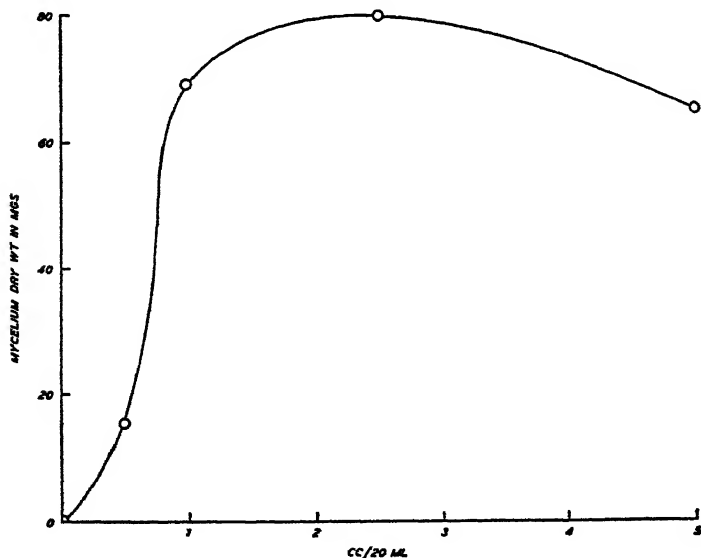
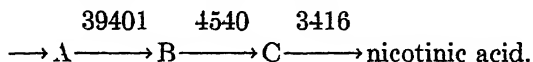


FIG. 2

Growth of Strain 39401 on Culture Filtrate of Strain 4540

is not nicotinic acid, as all three strains have similar nicotinic acid requirements and 4540 culture filtrates do not stimulate growth when inoculated with either 4540 itself or strain 3416. The fact that strain 4540 produces a substance active for strain 39401 but inactive for strain 3416 would indicate that this substance is B in the scheme shown earlier, and that the probable sequence of blocked reactions is



The precursor activity of culture filtrates rapidly disappears if strain 4540 is cultured longer than 4-5 days. The disappearance of precursor activity occurs about the time that pigment formation appears, and cultures with intense coloration show little or no precursor activity. It is possible that the pigment represents a spontaneous polymerization of the precursor or a breakdown product from it. On the other hand, it may be a product of abnormal metabolism of the precursor. In any event, pigment production in strain 4540 appears to be closely connected with precursor production.

### *Isolation of Precursor*

In preliminary experiments on the isolation of the precursor formed by strain 4540 it was noted that the precursor is labile in solution. Autoclaving for 10 minutes at 15 pounds pressure results in about 50% destruction. Solutions may, however, be stored several weeks at temperatures of 2-3°C. Fractionation must, therefore, be carried out rapidly at relatively low temperatures.

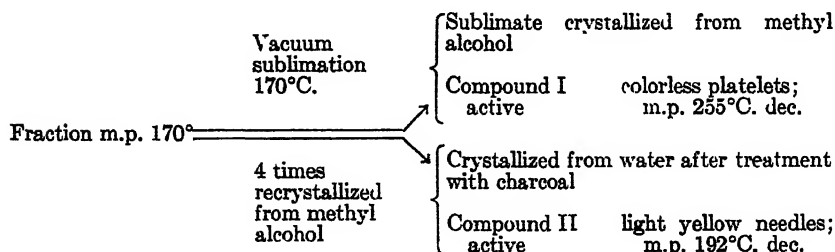
For isolation of the active material, strain 4540 is grown in 12 gallon pyrex bottles containing 40 liters of basal medium supplemented with 20 mg. of nicotinamide. Under these conditions nearly complete utilization of nicotinamide can be achieved without the addition of buffer which would be needed if the strains were grown on nicotinic acid. This concentration gives maximum precursor accumulation. The bottles are oxygenated by bubbling sterile air through the medium. Maximum precursor accumulation is reached in 6 days, the activity at that time corresponding approximately to that of 200 mg. of nicotinamide/40 l.

The fractionation scheme outlined in Table V yields two active, crystalline substances, both in small quantities. Substance I has an activity nearly equal to that of nicotinamide. Only about 10 mg. of this compound have been obtained to date. Elementary analysis, molecular weight and equivalent weight determinations suggest that

TABLE V

*Fractionation Scheme of Strain 4540 Culture Filtrate*

Treatment	Active Fraction
Culture filtrate shaken with Duolite C-3 <sup>-</sup> -hydrogen cycle	Adsorbed
Resin treated with 20% aqueous pyridine	Eluted—present in aqueous pyridine solution
Pyridine removed by concentrating <i>in vacuo</i> . Aqueous solution concentrated to 1–1.5 l.	
Ethyl ether extraction, continuous extractor. 3 days, with changes of ether twice a day	Ether soluble. This step gives 50% destruction of activity
Ethyl ether removed, remaining solid fractionally crystallized from methyl alcohol	Bulk of the activity crystallizes from a concentrated methyl alcohol solution as a mixed fraction, m.p. ca. 170°C.



\* Chemical Process Company, 58 Sutter Street, San Francisco, California

this compound, which contains no amino nitrogen, is a heterocyclic, monocarboxylic acid of either 6 or 7 carbon atoms. Final structure determination must await the isolation of more material. The activity of substance II is only about one-thousandth that of nicotinamide but appears to be real since repeated recrystallizations from different solvents do not alter the activity. Elementary analysis of the substance gives the following data:

	% C	% H	% N
Observed	57.55	5.68	8.44
Calculated for $C_6H_5NO_3$	57.48	5.39	8.43

Determination of the equivalent weight gives good agreement with this empirical formula and establishes the compound as a monocar-

boxylic acid. The structure of this compound has not yet been established. The physical properties of both compounds are similar and are such as to suggest that they are both oxypyridine carboxylic acids, substance II being a methylation product of substance I. Final determination of their structures must await further work.

### DISCUSSION

The immediate precursors in the biosynthesis of many of the vitamins may often be labile and, therefore, chemically rare or unknown substances. The properties of the nicotinic acid precursor, described earlier, suggest that it belongs to this category. The chance of identifying precursors of this type by testing known compounds is slight. It would seem likely that the structure of many such compounds can be determined by blocking specific reactions in such a way that the intermediates accumulate. Gene substitution, while not invariably leading to accumulation of intermediates, is one way of bringing about such specific blockage. As noted previously (6, 8, 9, 12, 13) as well as in the case of nicotinic acid, if gene substitution does lead to accumulation, the identification of the intermediate is greatly facilitated.

### SUMMARY

1. Five mutant strains of *Neurospora crassa* are described which require for growth nicotinic acid, nicotinamide or some related compound.
2. The activity of nicotinamide is independent of pH, while that of nicotinic acid shows a marked relation to pH. It is concluded that nicotinic acid enters the cell only as the undissociated acid. Correcting for dissociation of nicotinic acid, both substances have similar activity.
3. Genetic crosses and heterocaryon tests show that among these 5 mutant strains there are 3 different genetic types.
4. No known compound other than nicotinic acid or nicotinamide has so far been found to possess nicotinic acid-activity for these mutant strains.
5. When cultured on nicotinic acid or nicotinamide, strain 4540 accumulates a substance in the medium possessing nicotinic acid-activity for strain 39401.
6. Crystallization of two active substances from 4540 culture filtrates is reported. The empirical formula of one of these substances is

tentatively established as  $C_8H_9NO_3$ . The structure determinations of these compounds are not yet completed.

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# Physical and Chemical Studies on Southern Bean Mosaic Virus

## II. Crystallization by Dialysis

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### INTRODUCTION

Crystallization of plant viruses has usually been achieved through the use of ammonium or magnesium sulfates (1-10), but has also been accomplished with the aid of hydrophilic colloids (11, 12) and salt free media (9, 13), by acidification of sodium bicarbonate extracts (14, 15), and by high speed centrifugation in the presence (16) or absence (13) of salt. Rod-shaped viruses yield spindles (1-6) which are paracrystals (17), whereas spherical viruses yield dodecahedra (7, 9), octahedra (10), rhombic plates and prisms (8, 9, 11, 13, 16), hexagonal prisms (9) and various bi-pyramidal modifications (9, 12, 16).

Crystallization of a plant virus by dialysis is described in the present study. It was first observed during prolonged dialysis of southern bean mosaic virus against distilled water at room temperature. The yield of crystals forming was high and recrystallization by the identical procedure was readily carried out. The use of tap water instead of distilled water also resulted in crystallization of the virus.

It appeared that prolonged dialysis promoted crystallization by gradually removing dialyzable buffer and other electrolytes from the virus solution. As a result, the pH of the solution tended to approach that of minimum solubility of the virus, namely, pH 5.50, the isoelectric point (18), and favorable conditions for crystallization were made possible. Evidence for the importance of pH was afforded by the finding that the virus crystallized well when dialyzed against buffer at pH 5.50, but not against buffers at pH 5.00 and 6.00.

<sup>1</sup> Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

Crystallization by dialysis possessed the unique feature of producing material which was at the same time in the crystalline state and of low ash content. The more common salting-out methods, on the other hand, yield crystalline preparations which must of necessity contain large amounts of salt and from which the mother liquors are often not readily removed without danger of redissolving the crystals or of precipitating amorphous material. It was also of interest that crystallization by dialysis produced unusually large crystals, on some occasions 3-4 mm. in length.

Results strikingly similar to the above have been reported previously on the crystallization by dialysis of a globulin from milk (19) and of certain seed globulins (20).

It is possible that crystallization of other viruses and of other simple proteins may be accomplished by dialysis against water or against dilute buffers of appropriate pH. Success may be dependent, however, on suitable properties of the starting material, such as low solubility at the isoelectric point and sufficient tendency to form supersaturated solutions under these conditions.

## EXPERIMENTAL

### *Source of Virus*

Samples of virus employed in the crystallization studies were prepared by chemical and centrifugal fractionation procedures described elsewhere (16, 21). Preparations with which the detailed experiments to be described were carried out appeared completely homogeneous when subjected to electrophoretic test (18). Yields of crystalline virus represented, therefore, actual proportions of the total virus in the original samples, since measurements were not complicated by the presence of impurities. Concentrations of virus in different fractions were determined by the Kjeldahl method, and conversion of values for nitrogen to values for protein were made on the basis of 17.0% nitrogen in the virus (22).

### *Crystallization by Dialysis against Distilled Water*

Twenty-two ml. of southern bean mosaic virus solution, containing 30.6 mg. of virus/ml. in sodium phosphate buffer at pH 7, were transferred to a bag prepared from cellophane tubing and dialyzed at room temperature as follows. The bag, with contents, was first placed in a 250 ml. graduated cylinder through which a total of 30 l. of distilled water was slowly passed during a period of 3 days. Stationary dialysis was then carried out in a flask containing 6 l. of distilled water. Crystallization on the inner walls of the dialysis tube, observed the next day, was allowed to proceed for a day longer to bring it to apparent completion. The crust of crystals on the sides of the tube was broken by rotating the tube between the fingers. Microscopic examination revealed that no amorphous material was present in the preparation. The contents

of the dialysis tube were centrifuged and the heavy crystalline layer was washed 3 times by thorough mixing with 2 ml. portions of distilled water. When 0.1 ml. of 0.5 *M* disodium phosphate was added to the suspension, the crystals dissolved completely and instantaneously.

The original supernatant solution from the crystals contained 5.1 mg. of virus/ml. The proportion of virus which crystallized from solution was, therefore, 83%. The actual yield of crystals obtained was 48%. Fifteen per cent was recovered in the washings. The remaining loss resulted from the use of appreciable samples for microscopic study and other tests.

In a qualitative test, another sample of virus was found to yield a large amount of crystals when dialyzed against tap water instead of distilled water.

#### *Recrystallization by Dialysis against Distilled Water*

Five and nine tenths ml. of once-crystallized virus, containing 54 mg. of virus/ml. in approximately 0.01 *M* disodium phosphate buffer, were placed in a cellophane tube. The material was dialyzed against 30 l. of flowing distilled water as described above and then allowed to stand in a flask containing 6 l. of distilled water. Crystallization commenced after 2 days of the stationary dialysis and appeared complete by the third day. The product was separated by centrifugation, washed with 4 successive 2 ml. portions of distilled water, and dissolved in weak phosphate buffer. The proportion of material crystallizing from solution was 94%. The actual yield realized was 89%. Of the loss, 3.8% was recovered in the washings.

#### *Crystallization by Dialysis against Acetate Buffer at pH 5.5*

Twenty-four ml. of a pooled, somewhat pigmented sample of virus, containing 22.3 mg. of virus/ml. in phosphate buffer, were placed in a cellophane tube and dialyzed against distilled water as described previously. A very small crop of crystals resulted. The sample was then concentrated by pressure dialysis to about 0.4 its original volume to promote crystallization if possible. When this failed, the dialysis bag with contents was transferred to a flask containing 2 l. of 0.02 *M* sodium acetate-acetic acid buffer at pH 5.52. On the next day a heavy deposit of crystals was observed to have formed. Two days later crystallization appeared to be complete. The product was centrifuged, washed 3 times with 3 ml. portions of the acetate buffer, and finally dissolved in weak phosphate buffer. Nitrogen determinations revealed that 85% of the starting material had crystallized from solution. The actual yield realized was 69%. A loss of 4.4% was accounted for in the washings.

The amount of pigment relative to the virus present in the above preparation was actually too small to show up appreciably in moving boundary electrophoresis tests, yet it appeared sufficient to retard crystallization. Pigment has previously been reported to interfere also with crystallization of the virus by the use of salt (21). It was found in further studies that, by careful adjustment of the pH of the buffer, sufficiently concentrated preparations containing large amounts of pigment could be fairly readily crystallized. Under these conditions the pigment disappeared almost completely from the mother liquor but constituted a part of the crystals.

#### *Recrystallization by Dialysis against Acetate Buffer at pH 5.5*

Nine and eight-tenths ml. of virus, crystallized from acetate buffer and containing 36 mg. of virus/ml., were dialyzed in a cellophane tube against 2 l. of acetate buffer.



After 2 days, large crystals appeared on the walls of the tube and further crystallization proceeded over a 7-day period. The slow rate of crystallization may have been due to a relatively high pH of the buffer which in this instance was found to be 5.67, or to the fact that the phosphate buffer in which the starting material was dissolved was not removed before the dialysis against acetate buffer was carried out. The crystalline product was centrifuged, washed 3 times with 3 ml. aliquots of acetate buffer, and finally dissolved in weak phosphate buffer. The crystals separating from solution amounted to 76%; the final yield was 61%. The washings were not examined.

#### *Recovery of Crystalline Virus from Mother Liquors and Washings*

Supernatant solutions and washings of virus crystals from some of the various experiments were pooled and concentrated by pressure dialysis against acetate buffer. The concentration step was carried out in the refrigerator which effectively retarded the crystallization at this point since the virus became more soluble at the lower temperature (21). When the solution was then brought to room temperature, crystallization proceeded rapidly and, after dialysis was continued for 24 hours at the higher temperature, the crystalline product was separated and washed. The supernatant solution and washings were again concentrated and two further crops of crystals were obtained by repeating the above procedure. The amounts of starting material and percentage yields were not followed in these experiments; however, a final recovery of 137 mg. of crystalline product was realized with only 25 mg. of material remaining in the final mother liquor.

#### *Role of pH in Crystallization*

A preparation of noncrystalline virus was dialyzed against several liters of tap water to remove most of the electrolyte present. It was then divided into 3 samples which were placed in dialysis tubes and transferred to separate flasks containing 0.02 M acetate buffers at pH 5.00, 5.50 and 6.00, respectively. After 1 day, crystals appeared in the tube suspended in buffer at pH 5.50. Two days later, crystallization appeared complete at pH 5.50 but no crystallization occurred at the other pH values even after 4 days. To determine whether failure to crystallize at the outside pH values was due to an increased solubility of the virus under these conditions, the cellophane bag containing virus crystals at pH 5.50 was suspended in the flask containing buffer at pH 6.00. It was observed that the crystals gradually dissolved over a period of a few hours, thus providing evidence to explain the role of pH in crystallization.

At pH 5.00, most of the brown pigment present as an impurity precipitated from solution. This finding may prove valuable for the removal of pigment during purification of the virus.

#### *Properties of Crystals Obtained by Dialysis*

Crystals which separated during dialysis were often quite large and their outlines were readily visible to the naked eye. On some occasions crystals 3-4 mm. in length were obtained. The large crystals tended to be fragile, however, when placed under a cover-glass on a microscope

slide, and, therefore, only smaller ones could be photographed satisfactorily.

The various modifications of rhombic plates and prisms which were obtained, illustrated by the photomicrographs shown in Fig. 1, were similar to the rhombic prisms and bipyramids obtainable by crystallization of the same virus by means of high speed centrifugation in the presence of ammonium or magnesium sulfates (16, 21). Also similar to the latter, the present crystals showed no birefringence under the

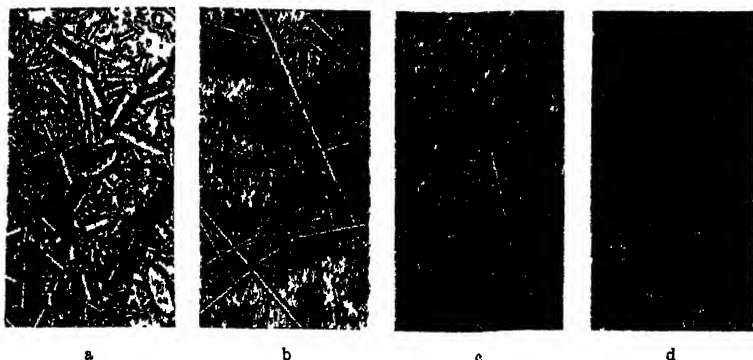


FIG. 1

Crystals of Southern Bean Mosaic Virus Obtained by Dialysis

a. Crystals separating on dialysis of a highly purified preparation against distilled water; magnification, 196 $\times$ .

b, c. Crystals formed rapidly on dialysis against acetate buffer at pH 5.52; magnifications, 96 $\times$  and 410 $\times$ , respectively.

d. Crystals formed slowly on dialysis against acetate buffer at pH 5.67, the smaller, coal-like particle representing a fragment broken from a very large crystal; magnification, 96 $\times$ .

polarizing microscope. This fact indicated that the crystals belong to the regular system. Their superficial appearance suggested, however, the orthorhombic, or possibly, the trigonal system. A measurement of interfacial angles will be necessary for solution of this problem.

When crystals in suspension were mechanically pressed against the inner surface of their container, they appeared glutinous. This probably indicated the hydrated character of the crystals, or perhaps of the virus particles within the crystals since the latter themselves have been demonstrated to contain considerable water of hydration (22).

Crystals placed in glass containers or on microscope slides, exhibited a marked tendency to redissolve. This is indicated by the rounding of the corners of the crystals shown in Fig. 1c. It may have been due to the leaching of traces of alkali from the glass.

The identity of the crystals with the virus itself was rendered probable by the high yield of product obtained from starting material which already had been established as homogeneous virus, by moving boundary electrophoresis experiments (18), by elementary analyses (22), and, finally, by activity measurements which revealed the infectiousness of the material.

### SUMMARY

Crystallization of southern bean mosaic virus to yield various modifications of rhombic plates and prisms was observed during dialysis at room temperature against media of distilled water, tap water and acetate buffer at pH 5.5. The pH of the dialysis medium and the purity of the starting material were found to be important to the crystallization process. Yields of crystals as high as 89% were obtained, and recrystallization was readily carried out. The method, in contrast to salting-out procedures, yielded a product which was at the same time crystalline and of low ash content. A tendency of the crystals to dissolve when placed in contact with glass and their glutinous character when mechanically pressed against the inner surfaces of their containers were noted. The identity of the crystals with the virus protein itself was established.

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# Physical and Chemical Studies on Southern Bean Mosaic Virus

## III. Electrophoretic and Nucleic Acid Studies

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### INTRODUCTION

Physical constants of southern bean mosaic (SBM) virus, such as sedimentation rate, diffusion rate, intrinsic viscosity and partial specific volume (1), have helped to differentiate it from other viruses. Electrophoretic mobility, like other physical constants, is also a fundamental property of viruses and thus aids in their characterization. The present work is concerned with the electrophoretic mobility of SBM virus as determined by the moving boundary method. From the data obtained, it was possible to deduce the pH range of electrochemical stability of the virus, the relationship between its electrophoretic mobility and pH, and its isoelectric point.

After the pH range of electrochemical stability had been established, preliminary studies were made of the decomposition products obtained outside this range to determine whether or not nucleic acid had been liberated. These studies were extended to see whether nucleic acid would also be liberated by heat denaturation.

### METHODS

Purified noncrystalline SBM virus was isolated from infected plants by chemical fractionation and high speed centrifugation (2). Crystalline virus was prepared by dialysis (3).

For electrophoretic studies, the following univalent buffers were made up at 0.02 ionic strength and were used as solvents for the purified virus: glycine-NaCl-HCl at

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<sup>1</sup> Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

pH 1.97, sodium chloroacetate-chloroacetic acid at pH 2.92, sodium acetate-acetic acid at pH 3.99 to 5.83, sodium veronal-HCl at pH 6.43 to 9.48, and glycine-NaCl-NaOH at pH 10.08 to 12.45. Samples of virus at concentrations of 3-5 mg./ml. were dialyzed overnight against the buffer chosen. Measurements of pH were made with a glass electrode pH meter and appropriate corrections were applied to readings taken in the stronger alkaline buffers. Electrophoretic mobilities were determined at 0°C. in a Tiselius apparatus (4) utilizing the Longworth scanning method (5) for photographing the boundaries. The mobility of the virus was taken as the average observed in the ascending and descending limbs.<sup>2</sup>

Preliminary sedimentation studies of the virus at pH 1.2 were carried out in a Bauer and Pickels type air-driven ultracentrifuge (7) equipped with a Svensson optical system (8).

Nitrogen determinations were made by the Kjeldahl method and phosphorus determinations by the King method (9).

### DISCUSSION OF EXPERIMENTAL RESULTS

At pH 2.92 to 11.50, the virus exhibited a single electrophoretic component, exemplified by the diagrams obtained at pH 7.38 which are shown at the top of Fig. 1. The limiting pH for electrochemical stability in acid solution was about the same as that for stability of infectivity; in alkaline solution, however, the pH range of electrochemical stability was much wider than that for stability of infectivity. As shown previously (10), purified virus became inactive when held for one hour at 27°C. at pH 8.3 or above; after 4 days at 3°C. it was inactive at pH 2.4 or below and at pH 9.3 or above. Apparently, the virus may lose its infectivity at certain hydrogen ion concentrations before it becomes disrupted.

The curvilinear relationship between pH and mobility of SBM virus is presented graphically in Fig. 2. The smooth curve drawn through the experimental points shows the isoelectric point to be 5.50. Noncrystalline and crystalline preparations were found to possess similar electrophoretic homogeneity and, as shown in Fig. 2, the same mobilities in the neighborhood of pH 4.0 and pH 7.4 where both were tested. The isoelectric points of tobacco mosaic and tomato bushy stunt viruses, determined by the same method, have been reported as 3.49 and 4.11, respectively (11, 12).

At pH 1.97 and 12.45, SBM virus showed multiple electrophoretic boundaries. The boundaries are indicated in the corresponding dia-

<sup>2</sup> In the presence of a "δ" effect (6), only the descending limb was considered. Actually, no appreciable "δ" boundary was observed between pH 2.92 and pH 11.50; only at pH 12.45 was there a marked "δ" effect.

grams of Fig. 1. Components in the acid medium migrated at rates of  $+0.80 \times 10^{-4}$  and  $+0.89 \times 10^{-4}$  cm./second/volt/cm.; those in the alkaline medium, at  $-0.63 \times 10^{-4}$  and  $-1.86 \times 10^{-4}$ . None of the values fitted the curve of Fig. 2. It was concluded, therefore, that the virus was unstable in solutions of this acidity or alkalinity and that complete changes in the electrophoretic properties had taken place.

Preliminary sedimentation measurements carried out with the virus at 5 mg./ml. in HCl at pH 1.2 also revealed two components, which

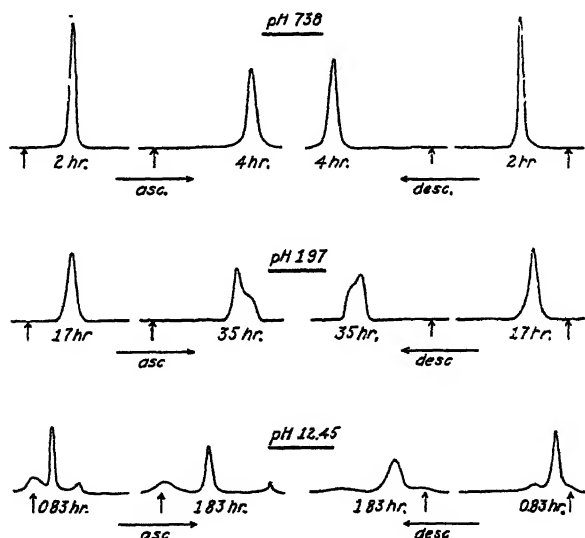


Fig. 1

Tracings of Longworth Scanning Diagrams of Ascending and Descending Electrophoretic Boundaries of Southern Bean Mosaic Virus

pH 7.38. Field strength, 3.8 volts/cm.

pH 1.97. Field strength, 2.6 volts/cm.

pH 12.45. Field strength, 2.3 volts/cm.

had sedimentation constants of 59.3 and 7.5 *S* in a first test and 58.8 and 11.9 *S* in a second. It was evident from this that the particles were partially split in acid solution, since unchanged virus at 5 mg./ml. sediments at a rate of 114 *S* (1). Sedimentation studies at the alkaline pH were not carried out. Other evidence, however, such as complete loss of the light scattering property of the virus at pH 12.45, indicated an extensive breakdown of the original particles under these conditions.



Further physical investigations on the nature of the split products obtained in acid and alkaline media were not made in detail. It appeared in this connection, however, that the component which migrated the fastest and occurred in the smallest proportion in the electrophoresis experiment at pH 12.45 may have represented a portion of the nucleic acid of the virus, since a number of other plant viruses have been shown to release nucleic acid in alkaline solution (13-19). A few chemical experiments were, therefore, carried out to provide additional evidence on this question.

Two ml. samples of virus solution containing 3.12 mg. of virus/ml. in 5% NaOH were prepared and allowed to stand for periods of time

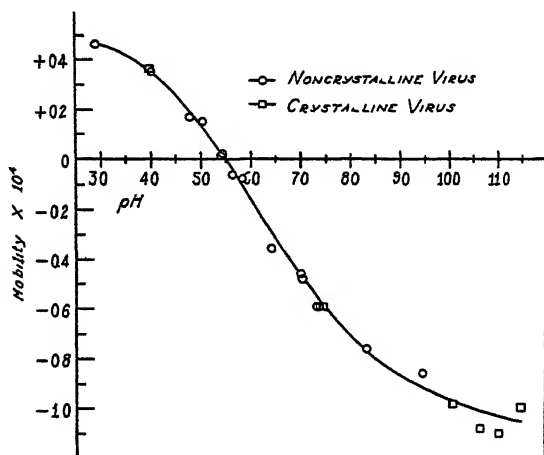


FIG. 2

Mobilities and Isoelectric Point of Southern Bean Mosaic Virus

varying from 2 to 100 minutes. The reactions were carried out at room temperature; hence, some decomposition of nucleic acid itself (20) may have resulted. Such decomposition, however, was not expected to interfere with the determinations, which involved only measurements of phosphorus. The solutions were then made acid to litmus with 0.18 ml. of glacial acetic acid to precipitate the denatured protein which was formed. The suspensions were diluted to 8.0 ml. and the precipitates were removed by centrifugation. Aliquots of the supernatants were analyzed for phosphorus as an index of nucleic acid and also for

nitrogen in order that nitrogen-phosphorus ratios might be calculated. The results, presented in Fig. 3, showed that all the phosphorus was liberated in a period of 60 minutes. The maximum phosphorus value obtained, 1.9%, accounted for all of this element known to occur in the virus (1). Simultaneously, however, the liberation of nitrogen proceeded abnormally rapidly and continued at an appreciable rate after all the phosphorus had been set free. The atomic ratio of nitrogen to phosphorus was about 6.2 during the first 60 minutes, but increased to 7.5 after 100 minutes. The atomic ratio of nitrogen to phosphorus in the nucleic acid of other plant viruses (14, 15, 16, 17, 18, 21) is around

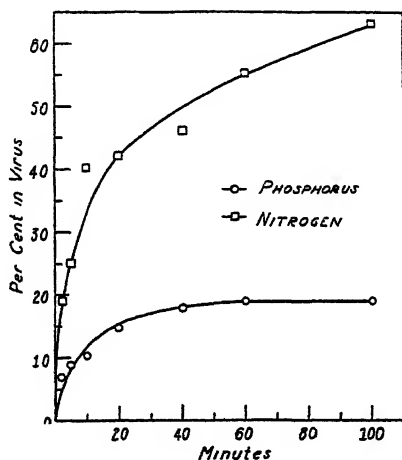


FIG. 3

Rate of Liberation of Phosphorus and Nitrogen from Southern Bean Mosaic Virus by 5% NaOH at Room Temperature

3.7. It is apparent, therefore, that the breakdown of the virus by alkali caused the formation of soluble nitrogen-containing fractions in addition to nucleic acid. The rate of cleavage of phosphorus from SBM virus by alkali appeared much slower than that from tobacco mosaic virus (13) and was similar to the rate of cleavage from tomato bushy stunt virus (15).

When heated to 100°C. for 1-4 minutes at a concentration of 1.56 mg./ml. in 0.1 M NaCl and 0.001 M acetate buffer at pH 5.53, the virus yielded a heavy coagulum with a slightly cloudy supernatant. Analyses for phosphorus made on the supernatants indicated the

presence of only traces of nucleic acid. The behavior of the virus here was again different from that of tobacco mosaic virus, from which nucleic acid may be set free by boiling in the presence of electrolyte (21), but similar to that of bushy stunt virus, in which case the nucleic acid clings to the denatured protein coagulum when the virus is heated in water followed by subsequent addition of electrolyte (22).

Although actual isolation of nucleic acid was not attempted, the results described indicate, by analogy to those obtained with other viruses, the presence of nucleic acid in the SBM virus particle. The average phosphorus content of plant virus nucleic acids isolated by other investigators already mentioned was calculated to be 8.9%. On this basis the phosphorus content of SBM virus corresponded to the presence of 21% nucleic acid in the virus.

#### SUMMARY

Electrophoretic studies on southern bean mosaic virus revealed a single component possessing an electrochemical stability range at 0°C. of pH 2.92–11.50 and an isoelectric point of pH 5.50. Outside the stability range, at pH 1.97 and 12.45, the virus was split into multiple components with abnormal mobilities. Sedimentation studies also revealed that the particles in the acid solution were split into components with sedimentation constants of the order of 59 *S* and 10 *S*, as contrasted with a sedimentation constant of 114 *S* for the intact virus.

Nucleic acid studies demonstrated that the phosphorus, and, therefore, by inference, the nucleic acid, of the virus was completely set free after treatment for 60 minutes with 5% NaOH at room temperature. The simultaneous cleavage of nitrogen, also measured, indicated the formation of other soluble nitrogen-containing fractions besides nucleic acid. Heat denaturation of southern bean mosaic virus did not free the nucleic acid from the protein portion of the virus particle. The phosphorus content of the virus was calculated to correspond to the presence of 21% nucleic acid.

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# The Effect of the Variation of Ionic Strength on the Electrophoretic Analysis of Bovine Plasma

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## INTRODUCTION

It has become evident that ionic strength not only exerts a great influence on the electrophoretic mobilities of proteins, but also affects markedly the distribution of the protein concentrations in an electrophoretic analysis of a complex mixture of proteins such as plasma or serum.

Davis and Cohn (1) observed that the influence of ionic strength on the electrophoretic mobility of carboxyhemoglobin was of the same order as that of pH. Their experiments were carried out at 25°C. Svensson (2) has found that, when phosphate buffer is used, the percentage component distribution of swine serum varies significantly with the ionic strength. He found that with an ionic strength of 0.1 the apparent albumin content was 59%, while at an ionic strength of 0.4 the apparent albumin content was lowered to 43%. At the same time the apparent concentrations of  $\alpha$ -globulin and  $\gamma$ -globulin varied in the opposite direction, while the apparent concentration of  $\beta$ -globulin seemed to remain fairly constant throughout the range of ionic strengths used. Perlmann and Kaufman (3), investigating human plasma with veronal buffer, have found as did Svensson though not as strikingly that the apparent albumin concentration was decreased as the ionic strength was increased with neutral salt while the apparent  $\gamma$ -globulin concentration was increased. They were able to obtain similar results by varying the protein concentration, and came to the conclusion that the ratio of concentration of protein to that of salt influences the apparent distribution of proteins in the electrophoretic diagram. Cooper (4), in an investigation with swine serum and artificial mixtures of proteins using veronal buffer, was unable to show the great variation of ionic strength that Svensson had demonstrated using phosphate buffer. Cooper used electrophoretically homogeneous bovine albumin and horse globulin in his mixtures. He concluded that ionic strength in the limits he used, 0.1–0.4, exerted no significant influence upon the percentage composition of protein mixtures as obtained electrophoretically in veronal buffer.

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Inasmuch as comparative electrophoretic analyses of bovine plasma and serum have been made (6), it was decided to make a study of the effect of ionic strength on the electrophoretic analysis of bovine plasma and artificial mixtures of proteins using phosphate and veronal (sodium diethylbarbiturate) buffers. Since Ballou, Boyer and Luck (7) have found that phosphate ion itself influences the mobility of proteins appreciably due to interaction of the phosphate ion with protein, the ionic strength of the phosphate buffers used in this investigation has been varied in two ways. First the ionic strength was varied by varying the concentration of the phosphate ion; secondly the concentration of phosphate ion was maintained constant and the ionic strength varied by varying the concentration of neutral salt. It was necessary to vary the ionic strength of the veronal buffer by adding neutral salt since the solubility product of veronal permits only limited variation in ionic strength.

#### EXPERIMENTAL

The technic of analysis, methods of determining mobilities and percentage component composition of the plasma, and the method of determining the duration of electrolysis have been described in previous communications (5, 6). The concentration of the  $\delta$  and  $\epsilon$  anomalies in each case was based upon the total area, including that under the  $\delta$  and  $\epsilon$  anomalies of the electrophoretic patterns, whereas the concentrations of the other components are based upon the total area, exclusive of the anomalies. This is the same procedure that Svensson (2) used.

The general composition of the buffers has been described (5, 6). The ionic strength of the phosphate buffer, pH 7.7 was varied from 0.05 to 0.40. The phosphate-KCl buffer contained  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$  of ionic strength of 0.05 and the ionic strength due to KCl was varied from 0 to 0.25. The pH of the phosphate buffer did not change with ionic strength. The change in pH of the phosphate-KCl buffer with change in ionic strength was slight. The veronal-NaCl buffer had a pH of 8.6 and an ionic strength of 0.05 to 0.10 due to NaV and an ionic strength of 0 to 0.2 due to NaCl. The change of pH with ionic strength in the veronal-NaCl buffer was only slight. The physical constants of the buffers appear in the tables with the other electrophoretic data.

The plasma used in this study was dried pooled bovine plasma previously described (6). Solutions of plasma were also prepared for electrolysis as previously described (5, 6), the final concentration of protein being 2% as determined refractometrically.

The proteins used in the mixtures were crystalline bovine serum albumin, an electrophoretically homogeneous bovine fibrinogen, and an electrophoretically homogeneous bovine  $\gamma$ -globulin. Each protein was dissolved separately in the appropriate buffer and dialyzed. The concentration of protein in each solution was determined refractometrically. Just before the electrophoretic determination was

made, appropriate quantities of each solution were combined so that the protein in the final solution consisted of 60% albumin, 20% fibrinogen, and 20%  $\gamma$ -globulin with the total protein comprising 2% of the solution. The final concentration of the protein in the solution was checked refractometrically to be sure that the final protein concentration was 2%.

## RESULTS

The apparent percentage component distributions of whole plasma in phosphate buffer pH 7.7 at various ionic strengths are given in Table I together with data for the buffers used. Duplicate runs and triplicate runs for some are reported. The apparent percentage concentration of each component is given for both the ascending and descending boundaries. It is quite evident from the table that, with increase in ionic strength, the apparent concentration of albumin decreases while  $\alpha$ -globulin increases markedly and  $\gamma$ -globulin increases less markedly. The apparent concentrations of  $\beta$ -globulin and fibrinogen remain rather constant. The  $\delta$  anomaly decreases rather markedly with increasing ionic strength up to 0.2 and then remains fairly constant. There is little change in the  $\epsilon$  anomaly after it can be distinguished. There seems to be a difference in the apparent concentrations for the ascending and descending boundaries at the lower ionic strengths for albumin and  $\alpha$ -globulin. This difference decreases with increase in ionic strength, thus tending toward better symmetry. The other components do not exhibit this behavior as markedly. The albumin-globulin ratios, given in the last column, tend to decrease with increase in ionic strength.

The variation of the mobility of each component of bovine plasma with change in ionic strength of phosphate buffer is given in Fig. 1. With albumin and  $\alpha$ -globulin there is a decrease in mobility with increase in ionic strength. The mobility of  $\beta$ -globulin appears fairly constant. There is an increase in mobility of fibrinogen and  $\gamma$ -globulin with increase in ionic strength.

Table II shows the apparent percentage component distribution of whole plasma in phosphate-KCl buffer, pH 7.7 and various ionic strengths. The values for apparent *per cent* concentrations are given for the ascending and descending boundaries. Duplicate runs are recorded in most cases. As in the phosphate buffer, there is a decrease in the apparent concentration of albumin with increase in ionic strength. From ionic strength 0.15 to 0.30 there is a separation of the



TABLE I  
*Percentage Component Distribution of Whole Plasma in Phosphate Buffer pH 7.7*

$r/2$	$K \times 10^3$	$V/cm$	Time in minutes	Albumin		$\alpha$		$\beta$		$\phi$		$\gamma$		$\delta$	$\epsilon$	$A/G$
				Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.			
0.05	1.90	7.90	76	51.6	48.6	5.3	7.6	8.2	10.1	20.5	21.6	11.1	12.1	25.2		0.99
0.05	1.90	7.90	120	53.6	45.5	5.4	8.7	8.4	10.8	19.7	22.2	11.1	12.8	26.8		1.00
0.05	1.90	7.90	90	51.2	49.1	5.0	7.3	9.0	10.2	20.4	19.6	10.9	13.8	23.8		1.01
0.10	3.56	5.62	150	44.0	43.0	9.5	10.3	8.4	10.2	20.9	21.6	17.2	14.9	17.9	4.1	0.77
0.10	3.56	5.62	195	46.6	41.0	11.1	11.7	9.8	10.1	19.5	21.5	13.0	15.6	20.1	4.3	0.78
0.10	3.56	5.62	165	45.6	42.1	9.6	12.9	9.7	10.2	20.2	20.8	14.9	14.0	19.2	4.3	0.78
0.15	5.14	5.83	180	38.4	38.8	14.5	16.3	9.0	9.5	20.5	20.1	17.6	15.2	15.2	4.0	0.63
0.15	5.14	4.87	210	38.6	38.7	14.7	14.3	8.9	10.5	19.3	20.7	18.5	15.8	15.4	3.3	0.63
0.20	6.60	4.55	240	37.0	37.8	15.6	15.7	9.5	9.8	20.2	19.8	17.7	16.9	17.2	1.6	0.60
0.20	6.68	4.48	240	37.0	38.0	15.8	15.8	8.6	10.6	21.1	19.8	17.4	15.8	11.0	3.1	0.60
0.30	9.54	3.14	360	34.1	35.5	15.9	17.0	8.9	9.6	21.1	20.9	20.0	17.0	10.5	4.1	0.53
0.30	9.56	3.76	300	34.4	36.8	16.1	16.1	8.8	10.3	21.5	19.1	19.2	17.7	12.4	3.2	0.55
0.40	12.27	2.44	480	33.1	34.2	14.3	17.0	10.4	9.5	22.2	20.2	19.7	18.9	15.2	4.0	0.52
0.40	12.27	2.44	480	32.4	35.2	15.2	16.9	10.4	9.5	22.3	20.4	19.7	18.0	14.5	4.8	0.51

$\alpha$ -globulin into  $\alpha_1$ - and  $\alpha_2$ -components. While there is relatively little change in the apparent concentration of  $\alpha_1$ -globulin with increase in ionic strength, there is a fluctuation in the apparent concentration of  $\alpha$ -globulin with increase in ionic strength. There is a definite increase in the sums of the apparent concentrations of  $\alpha_1$  and  $\alpha_2$  with increase in ionic strength. The apparent concentration of  $\beta$ -globulin and fibrinogen remain fairly constant throughout the range of ionic strengths used. There is an increase in the apparent concentration of  $\gamma$ -globulin with increase in ionic strength. The  $\delta$  anomaly shows a

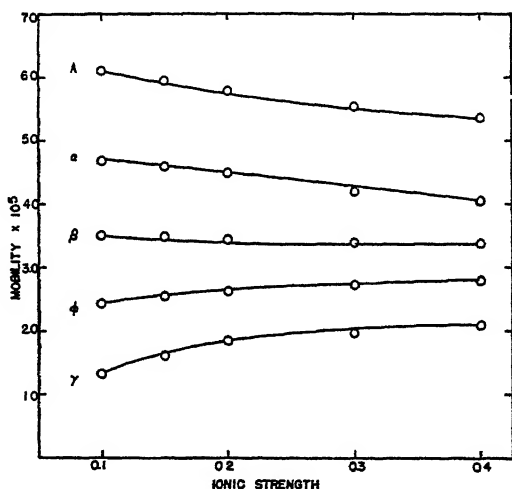


FIG. 1

Variation of Mobilities of the Components of Whole Plasma with Change in Ionic Strength of Phosphate Buffer pH 7.7

marked decrease in magnitude with increase in ionic strength while the  $\epsilon$  anomaly shows little variation. There is some variation between the apparent percentage concentrations for the ascending and descending boundaries of albumin at the lower ionic strengths. This behavior is exhibited somewhat by  $\alpha_1$ -globulin, but not to any extent by the other components. The values of the albumin-globulin ratio decrease with increase in ionic strength.

Fig. 2 shows the variation of the mobility of each component of bovine plasma with change in ionic strength of phosphate-KCl buffer.

TABLE II  
*Percentage Component Distribution of Whole Plasma in Phosphate-KCl Buffer pH 7.7*

$\Gamma/2$	Molarity of KCl	$K \times 10^3$	$V_{km}$	Time in min- utes	Albumin		$\alpha_1$		$\alpha_2$		$\beta$		$\phi$		$\gamma$		$\delta$	$\epsilon$	$A/G$
					Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.			
0.10	0.05	5.39	3.71	240	46.0	44.3			10.7	11.7	9.0	9.5	21.1	21.0	12.9	13.5	21.8	5.7	0.82
0.10	0.05	5.39	3.46	180	46.6	44.6			11.6	11.6	8.2	9.8	21.1	21.1	12.5	12.6	22.4	5.1	0.84
0.15	0.10	8.67	2.92	300	40.6	36.6	4.6	6.2	8.4	8.9	8.8	10.3	19.2	20.7	18.4	17.3	13.6	2.4	0.63
0.20	0.15	11.98	2.92	390	36.5	35.1	4.1	7.2	9.4	9.0	9.4	10.2	19.6	20.5	21.0	18.0	6.0	2.1	0.56
0.20	0.15	11.98	2.92	390	37.4	33.2	5.5	7.4	7.9	8.8	9.0	10.6	20.5	21.2	19.7	18.8	9.3	3.1	0.55
0.30	0.25	19.27	1.56	780	33.1	34.1	4.0	5.2	10.7	10.3	7.7	9.3	21.4	21.3	23.1	19.8	3.9	2.8	0.51
0.30	0.25	19.32	1.65	750	33.1	34.2	4.7	5.1	10.5	11.3	6.9	8.9	21.4	22.9	23.3	17.5	6.45	3.35	0.51

There is a small decrease of the mobilities of albumin,  $\alpha_1$ - and  $\alpha_2$ -globulins with increase in ionic strength. The mobility of the  $\beta$ -globulin remains fairly constant whereas the fibrinogen and  $\gamma$ -globulin show small increases in mobility with increase in ionic strength. It must be borne in mind that these values of mobilities of the components are determined in the presence of all the components of plasma and do not represent the values of pure proteins.

The apparent percentage component distributions of whole plasma in veronal-NaCl buffer are given in Table III. The amounts of NaCl

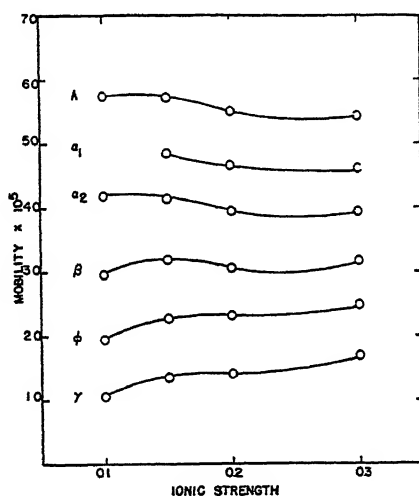


Fig. 2

Variation of Mobilities of the Components of Whole Plasma with Change in Ionic Strength of Phosphate-KCl Buffer pH 7.7

used for each ionic strength depends upon whether 0.05 or 0.10 *M* veronal buffer was used. Duplicate determinations are reported. Here again, the values of the apparent percentage concentration of each component are given for the ascending and descending boundaries. There is a decrease in the apparent concentration of albumin with increase in ionic strength, but not the marked decrease that was evident in the phosphate and phosphate-KCl buffers. The apparent concentrations of the  $\alpha_1$ -,  $\alpha_2$ -,  $\beta_1$ - and  $\beta_2$ -globulins and fibrinogen show little variation with increase in ionic strength. The concentrations of

TABLE III  
Percentage Component Distribution of Whole Plasma in Veronal-NaCl Buffer pH 8.6

$\Gamma/2$	Mo- larity of NaCl	K $\times 10^3$	V/cm	Time in min- utes	Albumin		$\alpha_1$		$\alpha_2$		$\beta_1$		$\beta_2$		$\phi$		$\gamma$		$\delta$	$\epsilon$	$\lambda/\delta$
					Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.	Asc.	Des.			
0.05	0	1.04	6.10	150	35.5	37.4	5.9	7.0	9.8	9.0	5.5	5.0	9.8	9.6	18.9	19.3	14.6	11.5	18.7	6.6	0.57
0.05	0	1.64	6.10	150	35.8	37.4	6.4	6.0	9.8	9.0	5.0	6.4	10.6	10.0	18.1	19.6	13.7	11.0	15.6	2.1	0.58
0.10	0	3.03	6.60	150	34.0	37.2	5.0	5.5	9.0	9.0	5.0	4.3	9.0	9.0	20.4	20.0	17.6	15.0	13.8	6.9	0.55
0.10	0	3.03	6.60	150	33.2	35.6	5.1	5.9	9.5	9.7	5.5	5.0	9.2	8.7	20.6	20.2	16.9	14.3	15.5	7.1	0.52
0.10	0.05	4.26	5.84	180	35.0	35.0	5.7	6.5	9.1	9.5	5.3	5.1	8.4	9.1	19.8	19.3	16.7	14.9	16.2	4.8	0.54
0.10	0.05	4.26	5.64	180	36.4	35.1	5.4	7.4	8.8	9.2	4.6	4.4	8.8	9.2	19.9	19.9	16.1	14.8	13.4	6.9	0.56
0.15	0.05	5.48	4.74	210	33.2	33.2	4.2	5.2	11.2	11.2	4.1	3.9	8.7	10.8	19.9	18.5	18.7	17.2	12.7	6.8	0.50
0.15	0.05	5.48	4.74	195	32.9	32.5	4.8	5.9	11.3	11.8	3.9	3.4	8.6	10.5	19.9	19.0	18.6	16.9	15.1	6.7	0.49
0.15	0.10	6.83	4.40	240	34.4	32.6	4.7	5.2	9.1	10.7	4.7	4.1	7.5	9.6	20.6	20.0	19.0	17.8	13.9	4.2	0.50
0.15	0.10	6.83	4.40	225	35.0	32.8	5.0	4.7	10.0	10.6	4.4	4.2	8.4	8.4	19.7	21.9	17.3	17.2	10.8	4.5	0.51
0.20	0.10	7.96	3.77	315	32.6	34.4	4.7	5.1	11.3	11.6	2.9	3.6	9.1	8.3	20.1	19.9	19.0	17.1	12.4	7.0	0.51
0.20	0.10	7.95	3.77	317	33.0	33.2	4.9	5.8	10.9	10.8	3.0	3.2	10.2	10.1	19.6	19.1	18.4	17.8	11.0	5.8	0.50
0.30	0.20	12.76	2.82	465	31.4	32.8	5.3	6.2	10.7	11.4	3.9	3.8	7.5	8.7	20.6	20.1	20.6	17.0	13.5	6.2	0.47
0.30	0.20	12.76	2.82	435	32.7	32.5	5.5	6.9	9.2	10.4	3.7	3.5	8.5	9.3	20.2	20.1	20.2	17.3	9.3	5.9	0.48

$\gamma$ -globulin shows a small increase with increase in ionic strength. The  $\delta$  anomaly tends to decrease with increase in ionic strength while the  $\epsilon$  anomaly remains fairly constant. There is some difference in the values for apparent percentage concentration in the ascending and descending boundaries for albumin at the lower ionic strengths. A similar difference, though small, exists for the two boundaries of  $\gamma$ -globulin throughout the range of ionic strengths. The differences that may exist for the other components are small and hardly seem significant. The albumin-

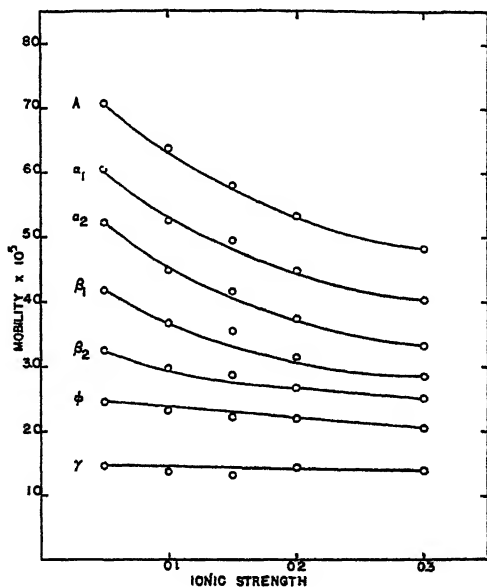


FIG. 3

Variation of the Mobilities of the Components of Whole Plasma with Change in Ionic Strength of Veronal-NaCl Buffer pH 8.6

globulin ratios show a tendency to decrease in value with increase in ionic strength, but not as markedly as in phosphate and phosphate-KCl buffers.

The variation of the mobility of each component in bovine plasma with change in ionic strength of veronal-NaCl buffer is given in Fig. 3. There is a marked decrease in the mobility of albumin with increase in ionic strength. This decrease in mobility with increase in ionic

strength grows progressively less for  $\alpha_1$ -,  $\alpha_2$ -,  $\beta_1$ - and  $\beta_2$ -globulins and fibrinogen. The mobility of  $\gamma$ -globulin remains fairly constant for the range of ionic strengths used, and in the presence of all the components of plasma.

The results of the electrophoretic studies of artificial mixtures of proteins in phosphate buffer at various ionic strengths are given in Table IV. Values for percentage component distribution for both

TABLE IV  
*Percentage Component Distribution of Artificial Mixtures  
in Phosphate Buffer pH 7.7*

r/2	K $\times 10^3$	V/cm	Time in min- utes	Albumin		$\phi$		$\gamma$		$\delta$	$\epsilon$	A/G
				Asc.	Des	Asc.	Des	Asc	Des.			
0.10	3.57	5.60	180	61.2	66.3	19.4	18.2	19.4	15.5	18.8	2.4	1.76
0.10	3.57	5.60	180	62.2	66.2	19.1	18.4	18.7	15.4	19.4	3.0	1.79
0.15	5.14	4.87	210	57.6	58.6	22.2	22.8	20.2	18.6	13.8	3.7	1.39
0.15	5.14	5.83	180	60.0	62.1	20.6	21.0	19.4	16.9	16.0	5.0	1.56
0.20	6.64	4.52	150	57.8	62.1	21.7	21.5	20.5	16.4	14.1	3.3	1.50
0.30	9.56	3.76	240	59.3	61.5	22.4	21.2	18.3	17.3	11.2	4.6	1.52
0.30	9.56	3.76	240	60.6	61.4	19.5	20.7	19.9	17.9	11.7	2.8	1.56
Av.				61.3		20.6		18.2				

ascending and descending boundaries are given. Duplicate determinations are recorded in most instances. The values are fairly constant for albumin, fibrinogen and  $\gamma$ -globulin. There is a small decrease in the  $\delta$  anomaly. The  $\epsilon$  anomaly is fairly constant. Although differences in the values of percentage concentration for the ascending and descending boundaries are larger for albumin and  $\gamma$ -globulin than for fibrinogen, they are probably within experimental error. The albumin-globulin ratios are a little high for ionic strength 0.10, but approach the theoretical (1.50) for the higher ionic strengths within experimental error.

The variation of the mobility of each component of the mixture with change in ionic strength of phosphate buffer is given in Fig. 4. There is pronounced decrease in the mobility of albumin with increase in ionic strength. The mobility of fibrinogen remains fairly constant throughout the range of ionic strengths used. On the other hand, the mobility of  $\gamma$ -globulin increases with increase in ionic strength.

The apparent percentage component distributions of the artificial mixtures in phosphate-KCl buffers for ascending and descending boundaries are given in Table V. The apparent concentrations of albumin, fibrinogen and  $\gamma$ -globulin are fairly constant throughout the range of ionic strengths used. With the exception of ionic strength 0.10, the  $\delta$  anomaly is rather constant throughout the experiment.

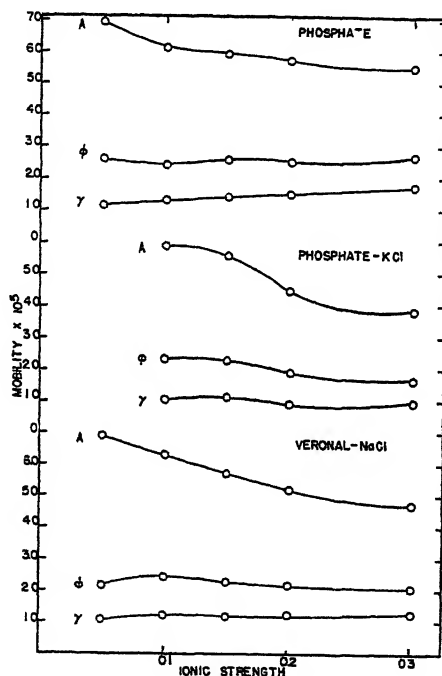


FIG. 4

Variation of the Mobilities of the Proteins in Artificial Mixtures with Changes in Ionic Strengths of Phosphate, Phosphate-KCl and Veronal-NaCl buffers

The  $\epsilon$  anomaly remains constant throughout the series of ionic strengths. There are considerable differences in the values of percentage concentration for the ascending and descending boundaries of all the components at the lower ionic strengths. These differences seem to decrease with increase in ionic strength, tending more toward perfect symmetry. The average of the values for the ascending and



descending boundaries give a rather consistent picture. The albumin-globulin ratios are fairly constant with the exception of the lowest ionic strength and approach the theoretical value of 1.50 favorably.

The change in mobility for each component in the mixture with change in ionic strength is given in Fig. 4. The mobility of albumin is significantly decreased with increase in ionic strength while the mobilities of fibrinogen and  $\gamma$ -globulin remain fairly constant throughout the range of ionic strengths used.

Table VI gives the apparent percentage component distribution of

TABLE V  
*Percentage Component Distribution of Artificial Mixtures  
in Phosphate-KCl Buffer pH 7.7*

$r/2$	Mo- larity of KC	$K \times 10^3$	$V/cm$	Time in min- utes	Albumin		$\phi$		$\gamma$		$\delta$	$\epsilon$	A,G
					Asc.	Des.	Asc.	Des.	Asc.	Des.			
0.10	0.05	5.39	5.57	180	66.4	63.5	14.5	19.5	19.1	17.0	24.1	1.8	1.85
0.15	0.10	8.63	3.48	241	56.6	61.1	17.2	21.8	26.2	17.1	5.8	2.7	1.43
0.15	0.10	8.63	3.48	240	57.8	60.4	14.5	21.5	27.7	18.1	4.9	2.7	1.46
0.20	0.15	11.97	2.92	390	60.6	61.8	14.3	17.9	25.1	20.3	4.0	1.9	1.58
0.20	0.15	11.97	2.92	390	58.4	60.0	15.8	18.7	25.8	21.3	7.2	1.8	1.45
0.30	0.25	19.30	1.55	780	58.5	60.5	19.5	19.4	22.0	20.1	2.7	1.1	1.47
Av.					60.5		18.0		21.6				

the artificial mixture of proteins in veronal-NaCl buffer for both ascending and descending boundaries. The albumin shows fairly constant values with the exception of the high values at ionic strength 0.05. The fibrinogen shows a fairly constant value throughout the range of ionic strengths used. The  $\gamma$ -globulin, on the other hand, has a tendency toward low values throughout the series. The  $\delta$  anomaly is rather high but has a tendency to decrease with increase in ionic strength. The  $\epsilon$  anomaly remains fairly constant. The difference in the values of percentage concentration for the ascending and descending boundaries are small, thus indicating a rather high degree of symmetry for all components. The values of the albumin-globulin ratio approaches the theoretical value of 1.50 with the exception of an abnormally high value at ionic strength 0.05.

In Fig. 4, it can be seen that the mobility of albumin decreases quite markedly with increase in ionic strength in the veronal-NaCl buffer. The mobilities of fibrinogen and  $\gamma$ -globulin remains fairly constant throughout the range of ionic strengths used.

TABLE VI  
*Percentage Component Distribution of Artificial mixtures  
in Veronal-NaCl Buffer pH 8.6*

1 2	Mo- larity of NaCl	K $\times 10^3$	$V/cm$	Time in min- utes	Albumin		$\alpha$		$\gamma$		$\delta$	$\epsilon$	A/G
					Asc.	Des.	Asc.	Des.	Asc.	Des.			
0.05	0	1.64	6.10	150	68.3	66.5	19.2	20.4	12.5	13.1	19.5	10.3	2.07
0.05	0	1.64	6.10	150	68.0	67.7	18.0	20.1	14.0	12.2	19.3	9.0	2.11
0.10	0.05	4.26	4.70	240	65.0	59.4	19.1	22.9	15.9	17.7	12.5	4.6	1.64
0.10	0	3.03	6.60	150	64.0	63.0	20.8	22.8	15.2	14.2	10.2	5.9	1.74
0.10	0	3.03	6.60	150	63.0	63.2	21.2	22.3	15.8	14.5	13.5	5.9	1.77
0.15	0.05	5.46	4.76	210	61.5	59.7	20.7	25.0	17.8	15.3	12.1	6.6	1.54
0.15	0.10	6.83	4.40	240	63.0	60.1	18.3	21.2	18.7	18.7	9.5	4.2	1.60
0.20	0.10	7.95	3.77	315	58.8	60.8	22.9	22.4	18.3	16.8	13.9	6.7	1.50
0.30	0.20	12.76	2.82	390	58.7	58.9	22.1	23.7	19.2	17.4	11.2	3.7	1.43
Av.					62.6		21.2		15.9				

## DISCUSSION

In the work of Longworth (8) in which he interpreted the results quantitatively according to Dole's (9) theory, it has been found that if disturbances due to convection and gravity are avoided, an initially sharp boundary between two solutions containing  $n$  species of ions will split, on passage of an electric current, into not more than  $n-1$  separate boundaries, each of which moves at a different rate. Using the fundamental equation of Longworth (8) for a moving boundary,  $\Delta T_j / \Delta C_j = V$ , in which  $V$  is the displacement of the boundary across which the differences of transference number and concentration of  $j$  ion are  $\Delta T_j$  and  $\Delta C_j$  respectively, the gradients in a given boundary due to a given ion are seen to have also superimposed on them gradients due to other ions in the mixture depending, of course, upon their relative mobility and concentration. Due to the large equivalent weight of the protein ions their contribution to the refractive index of the solution

is great as compared with their contribution to the conductance. In the ideal case, approached at high concentrations of buffer salt and low protein concentrations, the transference number of a given protein ion constituent is essentially zero and the gradients in the boundary due to this protein are not superimposed by gradients due to the other components. Svensson (2) has pointed out that these superimposed gradients are not entirely negligible and introduce errors in the analysis in actual electrophoretic patterns. He has pointed out that the fast components in a mixture of proteins such as serum are abnormally large at the expense of the slower components. In summarizing this theoretical concept, it should be possible to demonstrate a decrease in the fast components and a corresponding increase in the slower components of plasma with increase in ionic strength of the buffer salts; *i.e.*, decreasing the amount of current carried by the protein molecules.

When the ionic strength of phosphate buffer was varied by varying the concentration of the phosphate ion, the great decrease in albumin concentration with increase in ionic strength is accompanied by an increase in  $\alpha$ - and  $\gamma$ -globulin concentrations. There is a corresponding decrease in the size of the  $\delta$  anomaly. The concentration of fibrinogen and  $\beta$ -globulin remains fairly constant. This would indicate that there is existent an interaction between albumin and  $\alpha$ -globulin in phosphate buffer which is decreased when the ionic strength is increased. The increase in  $\gamma$ -globulin with decrease in size of  $\delta$  anomaly is due partly to the poor separation of  $\gamma$ ,  $\delta$  and  $\epsilon$  boundaries at the low ionic strength. In general these results parallel Svensson's very well.

When the phosphate ion was kept constant and the variation of ionic strength accomplished by the addition of neutral salt (KCl), results similar to those obtained with phosphate buffer alone were observed. The limiting values of the average apparent concentration of albumin,  $\alpha$ ,  $\gamma$  and  $\delta$  components, were reached more rapidly. The separation of the  $\alpha$  into  $\alpha_1$  and  $\alpha_2$  indicates again a depression of interaction by increase in ionic strength, and in this case increase of ionic strength by an increase in neutral salt while, at the same time, the relative concentration of phosphate ion is decreasing. When the concentration of  $\alpha_1$  was added to the concentration of  $\alpha_2$ , values of total apparent concentration of  $\alpha$ -globulin paralleled favorably the values for  $\alpha$  when the concentration of phosphate ion itself was varied and indicates an increase in total apparent  $\alpha$  concentration with decrease of apparent albumin concentration with increase in ionic

strength. This suggests not only an interaction between  $\alpha_1$  and  $\alpha_2$ , but also interaction between albumin,  $\alpha_1$  and  $\alpha_2$ . The separation of the  $\gamma$ ,  $\delta$  and  $\epsilon$  at low ionic strength was rather poor and can account, at least partly, for the respective increase and decrease of  $\gamma$  and  $\delta$  with increase in ionic strength. Fibrinogen and  $\beta$ -globulin remain fairly constant. These observations confirm Svensson's.

The striking characteristic of the data for veronal-NaCl buffer is the lack of the great variation that was experienced in phosphate buffers. The resolution of  $\gamma$ ,  $\delta$  and  $\epsilon$  was not complete at the lower ionic strengths thus accounting for low values of  $\gamma$  and high values

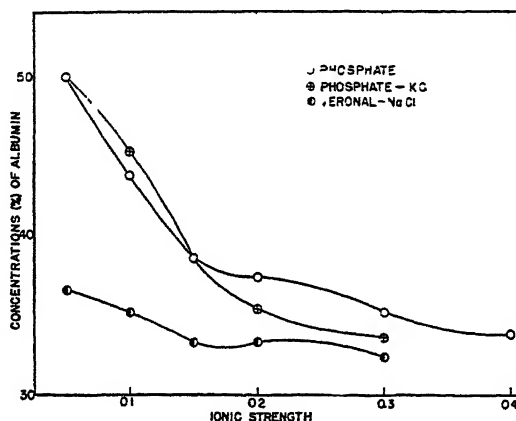


FIG. 5

Comparison of the Change in Apparent Concentration of Albumin in Whole Plasma with Changes in Ionic Strengths of Phosphate, Phosphate-KCl and Veronal-NaCl Buffers

for  $\delta$  and  $\epsilon$ . This finding is in agreement with the results of Cooper (4) who concluded that the apparent concentration of albumin in plasma showed no significant change when the ionic strength was increased with NaCl in veronal buffer.

Fig. 5 illustrates the comparative variation of the apparent concentration of albumin in whole plasma with change in ionic strength for phosphate, phosphate-KCl and veronal-NaCl buffers. It would appear that, in arriving at a theory for the influence of buffer salt concentrations on the electrophoretic analysis of protein mixtures, more than electrochemical behavior of the individual salts themselves

must be considered. The interaction of the proteins with themselves in the buffer and the interaction of the proteins with the buffer must be considered and emphasized. The phosphate-KCl curve approaches more nearly the veronal-NaCl curve while the phosphate curve approaches the former indicating that the phosphate ion is contributing to the interaction, but is depressed with increase in ionic strength and is more greatly depressed with neutral salt. The slow mobility of the veronal ion in contrast to the faster mobility of the phosphate ion according to Svensson's (2) theory can account for part of the differences but not all.

The variations of the average apparent percentage component distributions with variation in ionic strength when the artificial mixtures of proteins were examined in phosphate, phosphate-KCl and veronal-NaCl buffers do not appear to be great. Original mixtures consisted of 60% albumin, 20% fibrinogen and 20%  $\gamma$ -globulin. It can be seen that the greatest variation of albumin is at the lower ionic strengths, but even there the greatest variation is only 7% in veronal-NaCl buffer. From ionic strength 0.15 on, the values are certainly well within experimental error. The absence of  $\alpha$ -globulin in these mixtures no doubt contributes to the lack of variation of the apparent concentration of albumin with change in ionic strength in view of the evidence of its interaction with albumin in whole plasma. The  $\gamma$ ,  $\delta$  and  $\epsilon$  boundaries were poorly separated in the lowest ionic strengths.

In interpreting the whole plasma patterns no attempt was made to differentiate the portion of the  $\alpha$  that is not fibrinogen in view of the fact that this was previously done (6) and was not considered necessary for the purposes of the present investigation.

#### ACKNOWLEDGMENT

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#### SUMMARY

The variation of the apparent percentage component distribution of whole bovine plasma with variation in ionic strength in phosphate, phosphate-KCl and veronal-NaCl buffers has been studied.

The variation of the apparent percentage component distribution of

artificial protein mixtures with variation of ionic strength in phosphate, phosphate-KCl and veronal-NaCl buffers has been studied.

The findings of Svensson (2), namely, that the apparent concentration of albumin in swine serum in phosphate buffer decreases with increase in ionic strength when analyzed electrophoretically, has been confirmed in bovine plasma.

The findings of Cooper (4) that no real great variation in apparent concentration of albumin in swine serum exists with change in ionic strength of veronal-NaCl buffer when analyzed electrophoretically has been confirmed in bovine plasma.

While the electrochemical explanation is not to be minimized, the chemical interaction of proteins with buffers and with themselves must be held responsible, at least in part, for the variation observed.

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## Relation of Synthetic Folic Acid to Niacin Deficiency in Dogs \*

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### INTRODUCTION

It has been well established (1, 2) that a purified ration containing sucrose, casein, cottonseed oil and salts, and supplemented with the fat soluble vitamins A and D and the water soluble vitamins thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline will support excellent dog growth. In addition, it has been demonstrated that this ration is adequate for both growth and blood regeneration even in the presence of succinylsulfathiazole at levels up to 4% of the ration (3). It would seem, therefore, that the dog's requirement for additional factors could be demonstrated only under very specialized conditions. In a previous report (4) it was noted that the Norit eluate fraction from solubilized liver, known to be rich in folic acid, markedly enhanced the response to niacin in severely niacin-deficient dogs. This work has been repeated with synthetic folic acid instead of the crude Norit eluate.

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## EXPERIMENTAL

Weanling mongrel puppies were fed *ad libitum* the previously reported niacin-low synthetic ration (4). Adequate amounts (4) of thiamine, riboflavin, pyridoxine, calcium pantothenate, choline and vitamins A and D were given twice weekly. As a precautionary measure biotin was included at a level of 20  $\gamma$ /dog/day. Synthetic folic acid or *L. casei* factor (pteroylglutamic acid) was fed at a level of 25  $\gamma$ /dog/day unless otherwise indicated.

The usual symptoms of anorexia, drastic weight loss and diarrhea were used as criteria to denote severe niacin deficiency. In most cases the blood was examined for *per cent* hemoglobin, *per cent* hematocrit, and number of red cells/mm.<sup>3</sup> at the time of most severe deficiency and after recovery.

As the loss of dogs had been so great in the previous experiments (4) when the Norit eluate was not included, the effectiveness of the synthetic *L. casei* factor in these experiments was tested by removing it from the vitamin supplement after a dog had responded one or more times to standard 25 mg. doses of niacin. Each dog, therefore, served as its own control.

TABLE I

*Growth Response in Dogs Receiving Synthetic Ration plus Synthetic L. casei Factor, and the Blood Picture of Dogs before and after Response to Niacin*

Dog No.	No. of niacin depletions	Average response to 25 mg. niacin	Blood changes during one of the responses					
			Immediately before therapy			After response to niacin		
			Hemato-crit	Hemo-globin	Red cells	Hemato-crit	Hemo-globin	Red cells
		g.	per cent	g. per cent	millions/mm. <sup>3</sup>	per cent	g. per cent	millions/mm. <sup>3</sup>
A	5	1010	34	12.6	4.3	34	10.4	4.1
B	3	615	37	12.6	4.2	35	11.5	4.8
C	4	1500	40	13.7	5.3	35	13.6	4.7
D	2	1125	40	11.1	4.7	40	12.0	5.0
E	3	900	38	11.3	4.8	37	10.7	4.6
F	8	950	35	12.2	4.7	26	9.3	3.7
G	8	1000	27	10.0	3.5	29	8.8	3.6
H	6	1100	32	11.4	4.5	29	9.4	3.8
I	10	965	30	10.4	3.5	—	—	—

It is apparent from the data given in Table I that repeated niacin deficiencies can be produced and corrected when synthetic folic acid is supplied in the diet. The effectiveness of the Norit eluate factor previously demonstrated, therefore, was due directly to the folic acid which it contained. When folic acid is supplied, the niacin deficiency is not accompanied by a profound anemia (Table I) although the blood picture is not as satisfactory as that found in normal dogs (i.e., hematocrit 45%, hemoglobin 15.5 g./100 cc. and red cells 5.3 millions/mm.<sup>3</sup>). Since the blood picture did not improve during the growth response to niacin, it appears that the anemia is not directly related to the niacin deficiency. Although it is possible that the marginal level of niacin

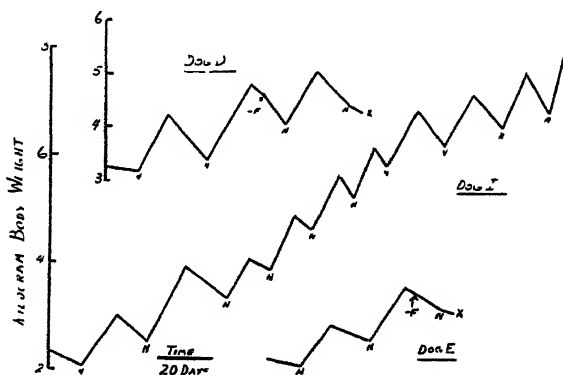


FIG. 1. Growth Response in Dogs Obtained from Niacin, with Folic Acid (Dog I) and without Folic Acid (Dogs D and E).

N = administer 25 mg. of niacin; -F = minus folic acid; X = death of dog.

supplied was insufficient for both growth response and blood regeneration it is more likely that other factors were limiting the hematopoietic response.

The repetitive character of the niacin deficiency which can be induced when synthetic *L. casei* factor is included in the ration is graphically shown in Fig. 1. (Dog I). The results obtained after the omission of the *L. casei* factor from the niacin-deficient diet are also shown in Fig. 1, dogs D and E, for the purpose of direct comparison. Dog D and dog J (not shown) responded once to niacin after omission of the *L. casei* factor, but failed to respond after the next deficiency, death ensuing. Dog D failed to respond to niacin at all after the *L. casei*

factor was removed. The niacin deficiency which occurs when *L. casei* factor is omitted is generally more profound, particularly with respect to the blood picture. While it was observed (Table I) that the niacin deficiencies, when folic acid was included, were not generally accompanied by severe anemias, there is a notable degeneration of the blood picture in the absence of *L. casei* factor. For example, dogs D and E (Fig. 1), just prior to death, had the following blood values, respectively: hematocrit, 17 and 12%, hemoglobin, 6.3 and 5.8 g/100 cc. and red cells 2.4 and 1.6 millions/mm.<sup>3</sup> Several attempts have been made to save the dogs showing this severe deficiency by administering folic acid, but all the dogs died except one. In this case 5 mg. of *L. casei* factor was given and the usual growth response to niacin resulted. Until many more trials are made it is difficult to conclude whether this response was due to the high level of folic acid used or to a less advanced stage of the deficiency.

Although folic acid clearly improved the biological response to niacin in the dog, evidence is at hand which indicates that additional factors may be involved in the niacin-deficiency syndrome. This evidence is based on the fact that, even with *L. casei* factor present, occasional dogs may give several normal responses to niacin and then fail rather suddenly, notwithstanding the fact that niacin is administered in ample quantities. Autopsy and analysis of the liver from two such dogs revealed liver damage and severely fatty livers (*i.e.*, 25 and 18% liver fat, respectively). The failure described is evident primarily in the younger dogs. Whether or not this deficiency is related to the hepatic damage in infantile pellagra reported in South Africa in humans (5) has not been ascertained.

Inasmuch as folic acid improved the response to niacin in niacin-deficient animals, experiments were conducted to extend the observation made by West (6) and repeated by Schaefer *et al.* (7) to the effect that sulfapyridine inhibited the usual curative effect of niacin in dogs rendered deficient on a blacktongue diet and that the feeding of fresh liver, together with the sulfapyridine, gave normal growth responses.

The inhibition by sulfa drug to niacin response was studied by administering the drug orally in 0.5 g. tablets three times daily after the dog showed definite niacin deficiency symptoms. (For the size of dog used, 3 tablets daily provided an adequate blood level of sulfa drug.) Niacin and various supplements were administered 24–36 hours after the sulfa drug administration was started. The effect of both sulfa-

pyridine and sulfathiazole were tested in this study. Two dogs, whose standard response to niacin had been 1000 and 900 g. respectively, were given sulfapyridine after niacin deficiency symptoms were evident. When niacin was then administered, no growth response occurred, but, as previously reported (6), when 150 g. of fresh liver was given to one dog, the usual good response was obtained. Dried, whole liver, 40 g., and vitamin C. 150 mg., failed to correct the sulfapyridine inhibition in the second dog, and not until fresh liver was given, did a growth response occur.

Since Cutting and Kuzell (8), and later Martin (9), indicated that sulfa drugs depressed the thyroid epithelium and acted generally to

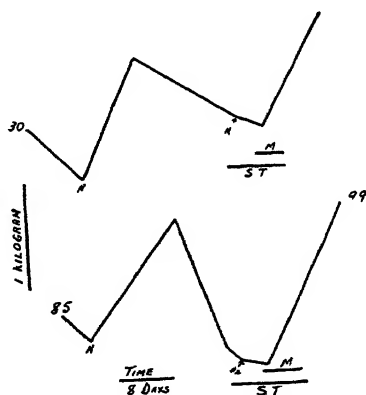


FIG. 2. Growth of Niacin-deficient Dogs Receiving Sulfathiazole and the Counteraction of Sulfathiazole Inhibition with Milk.

N<sub>1</sub> = niacin administration (25 mg.); N<sub>2</sub> = 50 mg. niacin; S.T. = sulfathiazole; M = milk, about 500 cc./day.

inhibit tissue metabolism, 1.2 mg. thyroxin was administered to another sulfapyridine treated dog which had failed to respond to niacin, but no positive result could be attributed to thyroxin and again fresh liver was resorted to in order to correct the deficiency. *p*-Aminobenzoic acid which is known to counteract the action of sulfa drug was tried at a level of 150 mg., but proved completely inactive.

Since Schaefer *et al.* (7) found sulfanilamide to be less effective than sulfapyridine in the inhibition of the response to niacin, the next experiments were made with sulfathiazole. The same experimental conditions as described above were maintained except that sulfathiazole replaced

sulfapyridine. Again a growth response in the deficient animals could only be elicited through the use of fresh liver. Since milk has always given excellent protection against blacktongue, despite its apparently low niacin content and because milk, as well as fresh liver, has been shown to provide a factor, or factors, important in the nutrition of the monkey (10), it was substituted for fresh liver in an attempt to correct the niacin inhibition induced by sulfathiazole. Typical results obtained with milk are shown in Fig. 2 and it is clear that milk adequately overcomes the sulfathiazole-induced inhibition. Although the dogs do not accept milk as readily as fresh liver, they soon start to drink it and then respond very well.

### DISCUSSION

Although sulfapyridine and sulfathiazole both inhibited the weight restoration normally induced by niacin, these drugs did not interfere with the alleviation of the usual niacin-deficiency symptoms. Whether or not large amounts of *L. casei* factor would prove effective in counteracting the effect of sulfa drug is not known, although enough of that compound seems to be present to prevent extreme anemias. The sulfa drug however, does tend to depress the hemoglobin level slightly.

The theory that sulfapyridine inhibits the synthesis of cozymase by the mechanism of competitive inhibition does not seem tenable, since sulfathiazole is as effective as sulfapyridine in inducing inhibition to niacin response in deficient dogs. On the basis of studies with bacteria (11), it seems more likely that sulfapyridine inhibits the function of the coenzyme. It is also evident from the observation of Handler (12) that the failure of dogs to gain weight when treated with sulfa drug may be due to anorexia, since anorexia was also produced in normal dogs on this regimen. This does not, however, explain the mechanism of the action of raw liver and fresh milk in counteracting the sulfa drug-induced anorexia. It appears more likely that liver and milk supply a specific factor, a deficiency of which has been produced by the conditions imposed.

### SUMMARY

Synthetic *L. casei* factor adequately replaces the crude "Norit eluate" in improving the response to standard doses of niacin in niacin-deficient animals. It also seems to play a role in maintaining a more adequate blood picture in these animals.

Fresh milk, as well as liver, is effective in counteracting the sulfathiazole-induced inhibition to niacin response in niacin-deficient animals.

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## LETTERS TO THE EDITORS

### Failure to Demonstrate Wrist Stiffness in Guinea Pigs Receiving Skim Milk Diets

Bahrs and Wulzen (1941) and Wagtendonk and Wulzen (1942-43) have described symptoms of wrist stiffness in guinea pigs receiving diets containing skim milk and apparently complete, and their cure or prevention by the administration of raw cream. An attempt to re-



FIG. 1

produce the condition with the diet of Wagtendonk and Wulzen (1942-43) failed, as, of 24 newly weaned guinea pigs weighing at the outset about 200 g., only 4 survived for longer than 32 days. No wrist stiffness was observed in any of them. The survivors were emaciated, unthrifty and bloated. Two, which lived 3 months, were killed and



autopsy revealed enormously distended caeca, atrophy of the intestine (Fig. 1) and marked fragility of the bones.

As it seemed possible that the mortality was due to the animals being too young at the start of the experiment, 3 of 6 guinea pigs which had been kept as controls on the stock diet were transferred on the 32nd day of the experiment to the skim milk diet. These animals lived longer than those given the experimental diet at an earlier stage, but in poor condition. Thirteen weeks after the beginning of the test one of these and one which had received the milk diet since weaning, were still alive. Acid phosphatase was measured in the serum from blood obtained by heart puncture from these 2 animals and from 2 controls. The values for the former were definitely lower. Raw cream (1 g. daily) given to one of the animals did not improve its condition nor raise the phosphatase titer.

TABLE I

*Values for Serum Phosphatase (King and Armstrong Units/100 ml.) of Guinea Pigs Receiving the Diet of Wagtendonk and Wulzen (1942-43) Alone (Group 1), Supplemented with 1 g. Raw Cream Daily (Group 2), or a Normal Stock Diet (Group 3)*

	Group 1		Group 2		Group 3	
	No. of animals	Serum phosphatase titer	No. of animals	Serum phosphatase titer	No. of animals	Serum phosphatase titer
Initial	8	28	8	26	7	28
After 6 weeks on diet	3	8	5	7	7	18
After 12 weeks on diet	No survivors		2	12	7	17

In a second experiment guinea pigs were allowed to reach a weight of 300-350 g., serum phosphatase was measured in the blood of all of them and they were divided into 3 groups of 8 guinea pigs with similar mean phosphatase titers. This time the diet described by Wagtendonk (1944) was used. Group 1 received the diet alone, group 2 with the addition of 1 g. raw cream, while group 3 had a normal stock diet. Animals in group 1 steadily lost weight and condition and none survived for more than 10 weeks. Most of those in group 2 behaved similarly and 6 of them died within 8 weeks. Two, however, suddenly improved and resumed growth. The improvement of one was only

temporary and it died after a few weeks while still receiving the cream; the other, whose supplement was stopped, continued to grow. Serum phosphatase determination showed very much lowered values for groups 1 and 2 (Table I). Of the two survivors in group 2 the one whose cream supplement was discontinued remained at this low level but the titer of the other rose to the normal level shortly before death.

Despite certain inconsistencies in our results our observations confirm the negative findings of Homburger and Reed (1945).

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National Institute for  
Research in Dairying,  
Shinfield, Reading.  
July 8, 1946.

S. K. KON  
M. J. BIRD  
M. E. COATES  
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E. E. SHEPHEARD

### Behavior of Phytofluene in the Animal Body

Phytofluene, a strongly fluorescing, colorless  $C_{40}$ -polyene, was recently found occurring in some vegetable materials used in human nutrition (1). Therefore, some experiments were performed to determine the behavior of phytofluene in the animal body.

Two rabbits were fed during a period of 4 and 2 weeks respectively, with 12 kg. and 6 kg. of commercial tomato paste containing about 15 mg. of phytofluene/kg. Most of the compound was destroyed but the liver of one animal contained 0.7 mg. Very small amounts occurred in the kidneys and spleens, approximately 0.01 mg. in each. The brains were free of phytofluene as was the urine (before and after acid hydrolysis). About 10% of the total amounts given appeared in the feces. No phytofluene could be detected in the liver of a rabbit previously fed on rabbit pellets. It would thus appear that, when large amounts of phytofluene are present in the diet, some may be absorbed and deposited in the liver. The major portion, however, is rapidly destroyed. Whether this destruction takes place in the gastro-intestinal tract is not clear from these tests.

It was reported recently that some commercial eggs were found free of phytofluene. Since, however, another batch bought on the market contained 1 mg. of the compound/kg. fresh yolks, we carried out the following experiment. During a period of a month 6 kg. of the tomato paste, mixed with "laying mash," was fed to two hens. Each of them laid 24 eggs, each of which contained 0.04–0.06 mg. of phytofluene, corresponding to 2.2–3.2 mg./kg. fresh yolk. No phytofluene was detected in the egg whites or in the hens' fat deposits. The livers contained about 0.1 mg. of phytofluene per liver, *i.e.*, 1.8 mg./kg. (fresh). Evidently, a little phytofluene may be introduced to the human body by hens' eggs under favorable conditions.

Strain (2) who had made basic observations on the occurrence of fluorescing material in plants, also reported that such substances (which were present in ingested carrots and presumably contained phytofluene) also appear in the butter. However, no fluorescing compound which is adsorbed below the  $\alpha$ -carotene zone was found in the egg yolks tested by this investigator.

The following materials were extracted and treated as had been described for vegetable products (1): livers, kidneys, brains, spleens and feces; before extractions the depot fat was dissolved in methanolic KOH at 50°C. The egg whites were treated as described below for the yolks.

Twenty-four egg-yolks were divided up and added slowly to 3.5 parts of methanol, stirred in a Waring Blendor. The suspension was twice mechanically shaken with one part of petroleum ether (b.p. 60–70°C.) and separated in a basket centrifuge. The residue appeared to be colorless. The intensely yellow solution (8.5 l.) was washed alcohol-free, concentrated *in vacuo* to 0.1 l. and shaken with 20% methanolic KOH for an hour, during which time a precipitate appeared. Acetone was then added until the precipitate dissolved. On cautious addition of water a precipitate reappeared but was then practically colorless. This was drained off from the bottom of the separatory funnel. The solution was washed alkali-free, dried over sodium sulphate, concentrated *in vacuo* to 50 ml. and re-saponified as described. The resulting petroleum ether solution (10 ml.) was then developed with the same solvent on a calcium hydroxide column (Shell brand lime, chemical hydrate, 98% through 325 mesh: 22 × 3.3 cm.). From the chromatogram the bottom section, which showed an intensely greenish-gray fluorescence in ultraviolet light, was cut out, eluted with alcohol, transferred into petroleum ether and estimated (1) on the basis,

$$E_{1\text{ cm.}}^{1\%} = 1200 \text{ at } 348 \text{ m}\mu.$$

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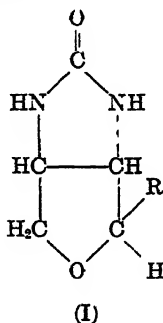
Gates and Crellin Laboratories,  
California Institute of Technology, Pasadena,  
(Contribution No. 1078), and Dept. of Biochemistry  
and Nutrition, Univ. of Southern  
California Medical School, Los Angeles  
July 18, 1946

A. SANDOVAL  
E. R. MESERVE  
H. J. DEUEL, JR.  
L. ZECHMEISTER

## On the Identity of Oxybiotin with O-Heterobiotin

Sir:

The first synthesis of a new class of compounds, namely, the hexahydro-2-oxo-1-furo-[3,4]-imidazoles of the general structure (I) was reported from our laboratories.<sup>1</sup>



Among the compounds described was the oxygen analogue of biotin, in which R represents  $-(\text{CH}_2)_4\text{COOH}$  and which we later called *dl*-oxybiotin.<sup>2</sup> Shortly after our announcement of the synthesis of oxybiotin Duschinsky *et al.*<sup>3</sup> reported the synthesis of an apparently similar compound without indicating either the method of synthesis or any intermediates involved in its preparation. These authors proposed the name *dl*-O-heterobiotin for their compound and demonstrated its high microbiological activity. *dl*-Oxybiotin was found to possess essentially the same biological activity as reported for *dl*-O-heterobiotin.

A direct comparison of *dl*-oxybiotin with *dl*-O-heterobiotin was made possible through the courtesy of Dr. A. J. Aeschlimann of the Hoffman-

<sup>1</sup> Hofmann, K., *J. Am. Chem. Soc.* **67**, 694 (1945).

<sup>2</sup> Pilgrim, F. T., Axelrod, A. E., Winnick, T., and Hofmann, K., *Science* **102**, 35 (1945).

<sup>3</sup> Duschinsky, R., Dolan, L. A., Flower, D., and Rubin, S. H., *Arch. Biochem.* **6**, 480 (1945).

LaRoche laboratories. When assayed with a variety of microorganisms (*S. cerevisiae* 139, *L. arabinosus*, *L. casei* and *S. fecalis*) the two compounds were shown to possess identical activity. The melting point of *dl*-O-heterobiotin (202–204°C.) was slightly lower than that of *dl*-oxybiotin (206–208°C.); however, a mixed melting point showed no depression. These facts establish the identity of the two compounds.

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August 6, 1946.

KLAUS HOFMANN  
A. E. AXELROD

### Deethylation of *N,N*-Diethyl-*p*-Aminoazobenzene\* in the Rat

Sirs:

Stevenson, Dobriner and Rhoads (1) have shown that *N,N*-dimethyl-*p*-aminoazobenzene (DMB) is demethylated during the course of metabolism by the isolation of the non-methylated derivative, *p*-phenylenediamine, from the urine of rats fed DMB. Miller, Miller and Baumann (2) have found that at least some of the demethylation occurs prior to the reduction and splitting of the azo linkage. These workers found *p*-aminoazobenzene (AB) in the tissues and blood of rats fed DMB.

A study of the metabolism of *N,N*-diethyl-*p*-aminoazobenzene (DEB) indicates that orally administered DEB is also dealkylated in the rat, and a distribution of AB in the tissues and blood has been found that is similar to that reported (2) when DMB is administered.

The methods used to extract and characterize DEB and AB are similar to those used by Miller and Baumann (3) in their studies on DMB. The compounds were identified by their behavior when chromatographed on aluminum oxide and by their absorption spectra in acid solution. DEB, in cottonseed oil, was mixed with a brown rice diet (6 mg. DEB/10 g. diet) and fed, plus carrot (4), *ad libitum*. The results are shown in the following table:

\* We wish to thank Dr. M. L. Crossley of the American Cyanamid Co. for the *N,N*-diethyl-*p*-aminoazobenzene used in these experiments.

*The Concentration of N,N-Diethyl-p-Aminoazobenzene (DEB) and p-Aminoazobenzene (AB) in the Tissues of Rats Fed DEB*  
 $\gamma$ \*\* of compounds found

Rat No.	Days on diet	Liver (5 g.)		Stomach contents (1 g.)		Cecum contents (1 g.)		Blood cells (1 ml.)		Serum (1 ml.)	
		DEB	AB	DEB	AB	DEB	AB	DEB	AB	DEB	AB
B-1	2	0	3	47	0	—	—	0	12	0	0
B-2	7	4	8	157	0	2	0	0	43	—	—
B-3	15	4	8	116	0	2	0	0	28	—	—
B-4	17	6	9	164	0	4	1	—	34	—	—
B-5	21	2	11	133	0	0	0	0	31	—	—

\*\* Zeros indicate that the compounds were not detected although small amounts (less than 1  $\gamma$ ) may have been present.

Small amounts of a third compound, believed to be *N*-ethyl-*p*-aminoazobenzene, have been found in the liver.

Thus, in the rat, both the carcinogen, *N,N*-dimethyl-*p*-aminoazobenzene, and the non-carcinogen (5), *N,N*-diethyl-*p*-aminoazobenzene, are to some extent dealkylated and yield *p*-aminoazobenzene.

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Memorial Hospital,  
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## Book Reviews

**Living Light.** By E. NEWTON HARVEY. 328 pp., 88 figures. Princeton University Press. Price \$4.00.

Dr. Harvey's "Living Light" is a rare example of a scientific treatise that can be read and enjoyed by many lay readers.

The reasons are, in the first place, the fascination of the phenomenon—the firefly has fired the imagination of many a poet since antiquity—and secondly, the fresh and simple way in which Dr. Harvey treats the subject in which he is one of the foremost experts. Accompanying the text are numerous illustrations, many of them the product of Dr. Harvey's own untiring and world-girdling effort in collecting and studying "living lights."

Beside providing interesting reading for naturalists and laymen alike, Dr. Harvey's book, we hope, will have a stimulating effect on the study of bioluminescence. While impressing the reader with the widespread occurrence of living light in the most varied types of animals, and with the large number of investigations which have been devoted to this phenomenon since the dawn of natural science, Dr. Harvey's book also shows clearly how little is as yet known about the physical and chemical mechanism of production of "animal light" and how few have been the applications of exact physico-chemical methods to this problem. From the chemical point of view, the discovery that in some species, the emission of light is associated with the autoxidation of a specific substrate, luciferin, by a specific enzyme, luciferase, has at least opened the field for fruitful research. The physical and physico-chemical approach, on the other hand, has yet to produce any significant results. (It must be stated in justice that our understanding of most chemiluminescences *in vitro* is equally poor: Only for a few simple cases such as the "cold flames" produced by sodium and halogen vapors, has a satisfactory explanation been developed.)

Because of this state of the field, the most valuable parts of Dr. Harvey's book are those dealing with the description of luminous organisms (Chapters I and II) and the chemistry of luminescence (Chapter IV), while Chapters III (Types of Luminescence) and VI (Physical Nature of Animal Light) contribute but little to the understanding of the phenomenon. Reading these chapters should spur new investigators to take up the study of "living light," in the hope that new progress can be achieved in this field by the use of more precise techniques and modern theoretical equipment.

The style of Dr. Harvey's book is refreshingly informal. Sometimes, it leans too much towards the colloquial. ("The author has kept some ostracods for twenty-one years which are still brilliant when moistened," p. 122.) Scientific precision suffers occasionally from this informality. ("Visible wave lengths frequently are present in such low intensity that it is impossible to measure them with any type of thermometer," p. 194.)

While a physicist or physical chemist may be disappointed by not finding in Dr. Harvey's book an attempt to analyze the observed phenomena of bioluminescence



more critically and on a higher physico-chemical level, and a purist may object to the informalities of his style, the most important fact remains that Dr. Harvey has given us an abundantly documented, fascinating account of a most interesting field of biology, and that his book has all the stimulating quality of a first-hand account, written by a man who had himself explored much of the field he is describing.

E. RABINOWITCH, Chicago, Ill.

**Chemical Spectroscopy.** By WALLACE R. BRODE. John Wiley and Sons, Inc., New York 1943 (second edition). 678 pages. Price \$8.00 (college edition \$7.00).

The second edition of this useful reference and textbook has been revised throughout. The general plan of the first edition has been retained, but new material has been added to the various chapters to such an extent that the total length of the book has been increased by about forty per cent. The additions have resulted in a more comprehensive treatment of many subjects; other subjects, such as fluorimetry, are included for the first time.

The emphasis of the book is upon the descriptive and experimental aspects of the subject, with special reference to applications to analytical chemistry. The theory of atomic and molecular spectra is discussed qualitatively with particular reference to those aspects of which an elementary understanding is a prerequisite to intelligent experimental work. Considerable space is devoted to the practical aspects of qualitative and quantitative emission spectrum analysis, and to absorption spectrophotometry.

As in the first edition, one section describes laboratory experiments based upon the author's experience in teaching a college course in chemical spectroscopy at the Ohio State University. These materially increase the usefulness of the book as a text.

Almost half the book is devoted to an extensive bibliography and to tables of emission lines, arranged with the needs of the practical worker in mind. Convenient conversion tables (wavelength—frequency, extinction—per cent transmission—logarithm of extinction) are also included.

The interests and needs of practical spectroscopists are so varied that an individual reviewing the book might be tempted to point out certain omissions, and to draw attention to subjects which, from the point of view of one's particular interests, appear to deserve more comprehensive treatment. Considered objectively, however, it would seem that the author has chosen material wisely, and that he has succeeded in stressing those aspects of practical spectroscopy which are deserving of greatest general attention.

This book should be a useful addition to the library of anyone concerned with the analytical use of spectroscopy or with the teaching of spectroscopic techniques.

JOHN R. LOOFBOUROW, Cambridge, Massachusetts

**Colloid Chemistry.** Volume VI, edited by J. ALEXANDER. 1230 pp., illustrated, Reinhold Publishing Corp., New York, N. Y. Price \$20.00

This is a very unusual book, the real value and significance of which is not easy to visualize and appraise. It is equally difficult to give the readers of this book reviewing section, in a comparatively short article, an idea of the character and content of Volume VI of J. Alexander's "Colloid Chemistry." The reviewer feels that the best

name for the book would be "Scientific Reader's Digest," because it exceeds the limits of "Colloid Chemistry" very substantially and even goes far beyond what may be considered the boundaries of colloid science in general. One has to stretch his imagination very far to consider articles on the "Mass Spectrograph" and on "Atomic Energy" as belonging into a volume of "Colloid Chemistry," particularly as the latter article includes systems of not directly colloidal character such as the earth, the sun, and the universe.

It seems to the reviewer that the real intention of Dr. Alexander was not so much to collect a series of articles on colloid chemistry, but much rather to collect a series of articles on generally interesting and actual problems written by outstanding authors. In this respect, the editor has completely succeeded, because the list of names which he has assembled as contributing authors is very distinguished and all subjects are attractive and suggestive.

Being that as it is, it will not surprise one that the character of the individual contributions is rather ununiform. There are several articles of considerable length (60-100 pages) which are written in the best style of a comprehensive and critical review paper, and there are others (2-5 pages) which do not contain more than a very brief sketch of the subject which they are supposed to treat. This heterogeneity in the scope of the individual contributions makes it difficult to discuss each of them (there are altogether 71 articles and 3 appendices) in this review and it may therefore be permitted to mention particularly the longer articles of typically colloidal content.

There is first a very extensive and interesting treatise of the "adsorption on the surface of solids and liquids" by W. D. Harkins and George Jura, which, in addition to a list of pertinent articles of the literature, contains a complete list of all publications by Professor Harkins and his school since 1916. There is next a similarly exhaustive description of the "Electron Microscope" by V. K. Zworykin and J. Hillier, which contains a very valuable list of applications of this instrument to various problems of colloid science. Of considerable interest is a contribution by E. H. Land and C. D. West on dichroic sheet polarizers, which gives a very clear and up to date description of this interesting new field of polymer science. Another of the longer articles, which seemed to the reviewer to be of special value is the contribution of C. K. Tseng on "Phycocolloids: Useful Seaweed Polysaccharides," which is a review paper of classical style.

In addition to these more exhaustive treatments, there are many shorter articles of great interest and clear and concise presentation, which cover the wide field of "General Principles and Specific Industries" (Part I), and "Synthetic Polymers and Plastics" (Part II). Some of them overlap each other to a certain extent, such as the two very well written articles on "Polystyrene"; one by Ivey Allen and L. Humphrey (pages 984 to 991) and the other by J. L. Auros (pages 992 to 1009); others appear to be somewhat too short, such as the article on "Casein Plastics" by H. V. Dunham (one page), or the article on "Acrylic Resins" by D. S. Frederick (three pages).

This brief extract of the content of this volume may serve to convey a preliminary impression of its character and of the editor's intentions. The book is very well edited and printed; it contains a large number of well organized tables and well selected figures and photographs. It will certainly help to spread the methods and ideas of colloid science and to advance their application in industry.

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# Microbiological Fat Synthesis by Means of *Rhodotorula* Yeast

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## INTRODUCTION

In recent years various authors have shown that, contrary to previous conceptions, it is possible to obtain yeast with a high fat content by aeration during cultivation (Nilsson, Enebo *et al.*, 1943; Rippel, 1943; Enebo, Elander *et al.*, 1944; Kleinzeller, 1944; Starkey, 1946). Suitable varieties of yeast and a method of cultivation adapted to high fat production are important conditions. Some species of *Rhodotorula* can be used for microbiological fat synthesis similarly to the use of *Torulopsis utilis* for microbiological protein synthesis under conditions of aerated growth. The term "fat yeast" seems suitable for species of yeast, such as *Rhodotorula gracilis*, which show special tendency to give rich fat production. This substance has been under investigation at this institution for some years back.

Certain fungi are known to have exceptionally high fat content normally. This fat-forming capacity has been studied in comparative detail, for example in *Endomycopsis vernalis*, certain species of *Aspergillus* and *Penicillium* and *Oospora lactis*. Usually, however, these fungi must be grown as surface cultures in flat dishes, which from the industrial standpoint is unfavorable in many respects. Of the mycelium-producing, fat-forming fungi, *Fusaria*, which may be grown on the surface or submerged (Nord *et al.*), deserves special mention, as it is particularly noteworthy that this organism may produce large amounts of fat from both pentoses and hexoses (1945).

The fat yield/g. of raw material, usually sugar, is a decisive factor in determining the applicability of a microorganism as a fat producer. Rippel (1940) introduced the term *fat coefficient* to express the number of g. of newly formed fat/100 g. of sugar consumed. He assumes a maximum fat coefficient of 15 as likely in yeast cultivation. We have, however, considerably exceeded this value in certain experiments, the difference apparently being due to the fact that Rippel did not envision such low protein contents as we encountered.

In earlier investigations with *Rh. gracilis*, using Kluyver flasks, Enebo *et al.* (1944) obtained a maximum of 43% fat in dry yeast and fat coefficients up to 15, but some cultures on a larger scale gave relatively low fat contents ( $\pm 20\%$ ) and correspondingly low fat coefficients (8-9).

The experiments reported in the present communication have resulted in considerable increases in the fat content and fat coefficient. Thus, in one series of cultivations (with *ca.* 51 l. of substrate each) we found 51-61% fat in the yeast and fat coefficients up to 18. The experiments have also shown a low growth rate during fat synthesis. This appears to be due to reduction in the N and P supplies, which is necessary for strong fat formation. When the additions of N and P are plentiful (which occasions insignificant fat formation) the growth can proceed as energetically as in the case of *T. utilis*. These circumstances may be illustrated by the mean generation time, which was *ca.* 3 hours with a plentiful N and P supply, 8-9 hours with moderate additions of these substances (20% fat), and 15-20 hours with small additions (60% fat). Hence, it appears that the extreme fat formation in *Rh. gracilis* must necessarily require a very long time. Continuous cultivation with small amounts of N and P only brought about an extremely slow increase. The following table of the most important cell components illustrates the difference between protein yeast and fat yeast.

TABLE I  
*Compositions of Protein Yeast and Fat Yeast*

	<i>T. utilis</i> *	<i>Rh. gracilis</i>
Protein (%)	59	13
Carbohydrate (%)	30	24
Fat (%)	3	60
Mineral substances (%)	8	3
	100	100

\* According to FINK, LECHNER & KREBS, (1939).

The values for *Rh. gracilis* apply to yeast cultivated with an eye to especially strong fat formation. They seem to represent almost the upper limit of fat formation and the lowest possible protein content for cultivation according to the procedure indicated below. (In individual cases we have obtained yeast with somewhat over 60% fat and down to 12% protein.)

Starkey has recently isolated a yeast from soil, which, when grown on nitrogen-free glucose-agar plates, produced cells containing 50-63% lipid, 20-25% of the

glucose used being converted to cell substance and the highest yield of glucose to fat being 15.6%.

The practical question arises as to the degree to which the *gracilis* yeast can be considered as an agent for large scale fat synthesis. The continuous production of fat yeast with 60% fat content in the same manner as protein yeast appears to be without economic prospects in view of the difference between the growth rates of the two varieties. If, however, a semi-continuous production of fat yeast were arranged so that, from a continuous growth generation, portions were tapped off continuously or discontinuously and "fatted" separately, a cultivation scheme would be obtained which, under certain conditions, appears worthy of consideration from the technical point of view. This procedure corresponds in principle to our cultivations in Kluyver flasks and in the large yeast-cultivation apparatus, as described on p. 387 (*e.g.*, experiments 31 and 32). By means of this division, the risk of infection characterizing continuous *Rhodotorula* cultivation is largely eliminated.

Another procedure is to carry out the fat synthesis in such a way that the product admittedly shows a relatively low fat content, but the yeast growth proceeds at such a rate that the production can proceed in a wholly continuous manner. Several suggestions of this type have been made in recent years. The dry yeast thus obtained is said to contain 15-30% fat and 15-35% protein.

Krohn (1944) suggested a method for the conversion of wood sugar into fat yeast and production was to have started in Germany. The fat content of the yeast was about 20% and the protein content over 50%. (It is difficult to reconcile these figures on the energy basis.) Details are not given as to the species of yeast and other important factors. In view of international events at the end of 1944 and the beginning of 1945, it is probable that this project was not realized in practice.

## EXPERIMENTAL

### *a. Method of Fat Estimation*

The fat content of the yeast was determined by a modification of the method of Otto & Hampel, based on treatment of the yeast with HCl followed by extraction (*cf.* Nilsson *et al.*, 1943 and Enebo *et al.*, 1944).

### *b. Composition of the Substrate*

It has long been known that the fat and protein contents of yeast are in some measure inversely proportional to each other. Hence, it

is possible to stimulate the fat formation by limiting the supply of the nutrients essential for protein synthesis, particularly nitrogen compounds. The phosphate supply apparently should also be less than in ordinary yeast cultivation.

As it had been proved that further reductions in the *nitrogen* and *phosphate* additions to the substrate employed in our previous experiments increased the fat content, we ended by using additions of 0.53 g. N (as  $(\text{NH}_4)_2\text{SO}_4$ ) and 0.46 g. P (as  $\text{K}_2\text{HPO}_4$ )/100 g. sugar (calculated as glucose). Further quantities of sugar, N and P were, however, introduced by adding 62.5 ml. of 20% beer wort/100 g. of glucose to the nutrient solution. The wort contained about 10 g. sugar, 0.10 g. N and 0.04 g. P/62.5 ml. Thus, the additions of N and P/100 g. total sugar were 0.57 and 0.46 g., respectively. In one isolated case (experiment 32, see (b)) only half the above-mentioned quantity of wort was added/100 g. glucose. This implied reductions in the concentrations of N and P in the substrate by 0.05 and 0.02 g./l., respectively.

The phosphate concentration which we used was relatively large in comparison with what has been considered suitable in experiments with other fat-forming organisms. In this case, however, we were concerned with avoiding further suppression of growth by inadequate supplies of mineral substances, as the growth in any case proceeded at a low rate during formation of fat.

As mentioned above, addition of *beer wort* brings about more rapid growth. An increase in the addition to above ca. 60 ml./100 g. sugar has proved, however, to be without effect. Nor is the growth accelerated if the substrate is provided with aneurin hydrochloride in a concentration of for example, 1000  $\gamma$ /100 g. sugar.

The replacement of ammonia-N by asparagine-N has also proved to be without effect on the growth rate.

*Rh. gracilis* splits disaccharides rather slowly, which may influence the growth rate when saccharose substrates are employed. The degree of splitting is dependent upon the pH, as shown by the following values obtained after cultivation for 24 hours on saccharose substrates.

TABLE II  
*Degree of Inversion at Different pH Values*

pH of substrate	Degree of inversion per cent
-----------------	---------------------------------

4.0	85
4.5	54
6.5	32

In order to facilitate the growth of the yeast we have, therefore, used invert sugar instead of saccharose. This had the additional

advantage that sugar determinations in the course of the cultivation were simplified.

In view of these circumstances, we employed the following nutrient solution in most of the experiments that form the basis of this communication:

	g./l.
Invert sugar	40.0
$(\text{NH}_4)_2\text{SO}_4$	1.0
$\text{K}_2\text{HPO}_4$	1.0
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	1.0
$\text{NaCl}$	0.5
$\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$	0.5
$\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$	0.005
+ 25 ml. 20% beer wort, pH to 4.5 with $\text{H}_2\text{SO}_4$	

\* Prepared by inversion of Svenska Sockerbolaget's commercial quality K5P with 1 ml. conc.  $\text{HCl}$ /l. of 47.5% solution.

In the cultivation of seed yeast in Kluyver flasks the additions of  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{K}_2\text{HPO}_4$  were increased to 5.0 and 2.0 g./l., respectively. This gave a higher yield of seed yeast. The fat content of the latter amounted to only 20%, so that it was better suited to reproduction than yeast very rich in fat.

### *c. Cultivation Experiments with High Fat Formation*

The yeast-cultivation apparatus was a vertical cylinder of acid-resistant steel (225 cm. high  $\times$  25 cm. diam.) provided with porous aerators immediately above the bottom. The top and bottom were removable to facilitate cleaning. Before use the apparatus was disconnected and the components washed with a sterilizing solution, followed by reassembly and steaming. To prevent frothing-over during the culture period, a rotating foam-suppressor, a cross of sheet iron pieces set edgewise (120 mm. diam.  $\times$  25 mm. high) mounted on a central vertical axle and driven by a  $1\frac{1}{8}$  h.p. motor @ 1400 r.p.m., was provided.

The substrate described in *b* was used in exp. 31. In exp. 32 the amount of nutrient salts was doubled. The sugar content was 70 g./l. and the wort addition was unchanged, i.e., 25 ml./l., temperature 27–29°C. Other experimental conditions and results are shown in Table III.

A similar experiment with 90 g. sugar/l. of nutrient solution was infected, so that the yeast yield was only 27% for a fat content of



53.1%. (The content of  $(\text{NH}_4)_2\text{SO}_4$  was 2.5 g./l. Otherwise as in experiment 32.) It is clear that relatively concentrated sugar solutions may be employed, which is of importance from the technical point of view.

The figures indicate that the process, as stated previously, consists of two stages, the *phases of protein formation and fat formation*. In

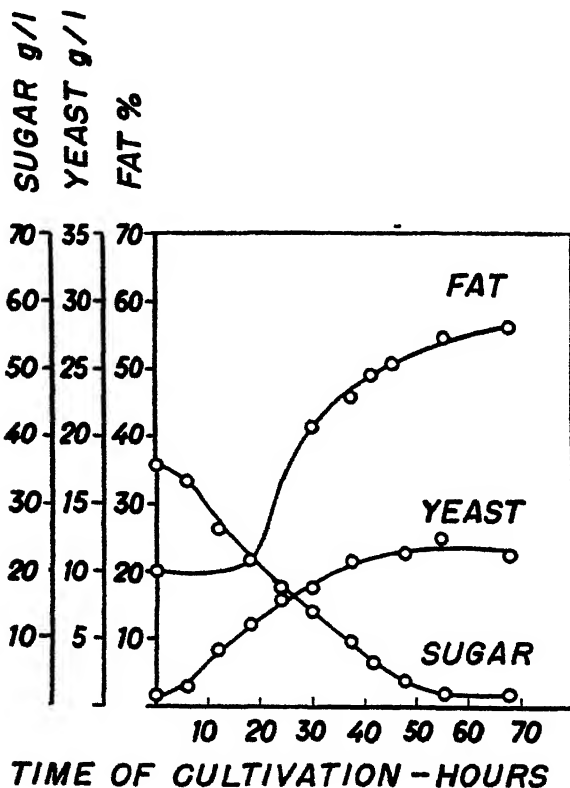


FIG. 1

Sugar Content, Quantity of Yeast and Fat Content as Functions of Time

experiment 31 the yeast quantity augmented at an increasing rate until 12.5 hours after initiation of the culture, after which the growth proceeded more slowly. After about 17 hours the true fat-formation process began. The sugar curve, however, had a constant slope, *i.e.*, the transition from the phase of rapid yeast growth and slow fat

formation to the opposite phase had no effect on the consumption of sugar.

The difference between the periods of growth and fat formation appears most clearly in the following Table from experiment 31, which shows the quantities of alkali necessary to maintain constant pH.

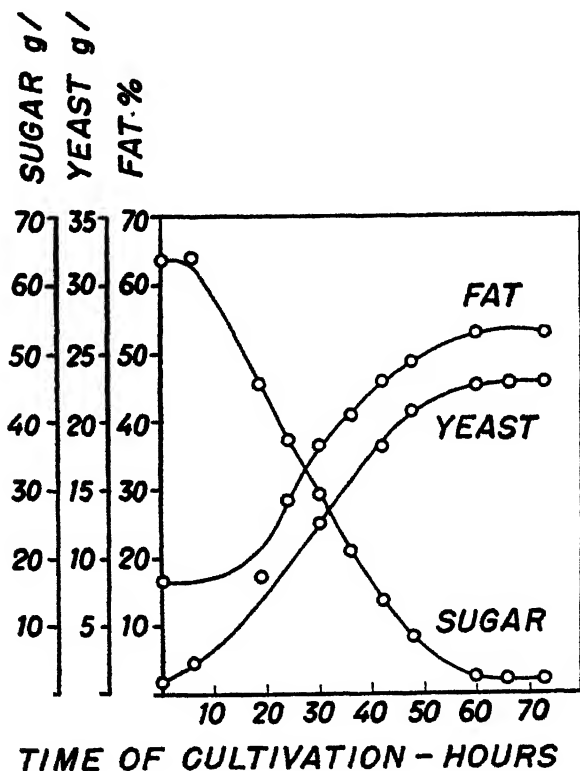


FIG. 2

Sugar Content, Quantity of Yeast and Fat Content as Functions of Time

When the ammonia-nitrogen is assimilated by the yeast, the pH shows a continuous decrease due to the disappearance of  $\text{NH}_4^+$  ions. As soon as the protein formation ceases, the consumption of alkali also ceases. Toward the end of the experiment the pH rose somewhat, so that the adjustment had to be made with  $\text{H}_2\text{SO}_4$ .

TABLE III  
*Values for Some Larger Cultivations*

	Experiment 31	Experiment 32
Time of cultivation (hr.)	68	73
Volume of nutrient solution (l.)	47.0	52.0
Seed culture: Volume (l.)	5.5	5.0
Yeast quantity (g. dry substance)	46.0	55.4
Fat content of yeast (%)	20.0	16.5
Total quantity of yeast (g. dry substance)	593	1190
Newly formed yeast (g. dry substance)	517	1135
Added sugar (g.)	1880	3615
Yield of yeast (%)	29.1	31.4
Content of crude fat in dry yeast substance (%)	56.6	52.2
Newly formed fat (g.)	326	663
Fat coefficient	17.3	16.9
Degree of reproduction	12.9	21.5
Number of generation	3.69	4.42
Generation time* (hr.)	18.4	16.5
N-content of yeast (%)	2.10	
Ash content of yeast (%)	3.5	3.6

\* The generation time  $t_g$  at the cultivation time  $t_{tot}$  is obtained as  $t_g = \frac{t_{tot}}{n}$

where  $n$  is the number of generations. If  $f = \frac{\text{total quantity of yeast}}{\text{added quantity of yeast}}$ ,  $2^n = f$ , or  $n = 3.32 \times \log_{10} f$ .

TABLE IV  
*Alkali Consumption during the Growth Period*

Time in hours	Alkali consumption (ml. 4 N alkali/hr.)
0	0
1	5
2	10
3	14
4	17
5	22
6	45
7	57
8	37
9	16
10	3

*d. The Minimum Quantity of Nitrogen*

The following series of experiments in Kluuyver flasks shows that, although the period of fat formation can be prolonged by addition of extra sugar, this is disadvantageous with respect to sugar economy. The nitrogen content of the substrate may not be less than 0.6 g./100 g. sugar.

One liter of standard substrate (sec *a*) with 40.0 g. glucose together with 6.0 g. citric acid was used in each flask. In experiment II, moreover, 0.5 g. of the ammonium sulphate was replaced by 0.57 g. asparagine (1 H<sub>2</sub>O). The seed yeast contained 20.3% fat. Temperature 27–29°C.

TABLE V  
*Effect of Excess Sugar Relative to N and P*

Experiment No.	Initial		Final		Yeast	Fat	
	Yeast	Sugar	Yeast	Sugar	Yield	Content	Coefficient
	g.	g.	g.	g.	per cent	per cent	
			(a)				
I	0.87	40.0	10.0	7.5	28.1	61.1	18.2
II	0.87	40.0	9.41	8.5	27.1	55.0	15.9
III	0.87	40.0	11.0	1.4	26.2	56.3	15.6
			(b) after further addition of sugar				
I	8.19	26.6	10.0	11.9	12.3	51.2	0.81
II	7.70	27.5	11.55	6.0	17.9	54.8	9.7
III	9.0	21.6	10.30	6.0	8.3	63.2	9.2

*Note.* To eliminate the necessity for continuous opening of the Kluuyver flasks to adjust pH, we added citric acid as buffer, as in a previous series of small-scale experiments. Citric acid may exert an inhibitory effect on divers microorganisms but did not do so in the present case, as shown by a special experiment. This was carried out with a relatively generous supply of nitrogen (as asparagine). The growth proceeded rapidly both with and without citrate (generation times 8.0 and 8.1 hours respectively). There is, however, the risk that the yeast will employ the citrate as a carbon source. This would influence the calculations of yield and fat coefficient, which are based exclusively on the amounts of sugar added or consumed. As, however, we did not attain higher yields in the Kluuyver-flask cultivations than in the larger experiments, this risk was presumably rather small, especially as the sugar was always present in excess. The addition of citric acid never exceeded 15% of the quantity of added sugar. When ammonia was replaced by asparagine the change in pH was negligible.

*e. The Consumption of Sugar during Fat Formation*

The yeast yield in these experiments, where alcohol formation is lacking and the excretion of proteins by the yeast seems extremely insignificant, may be formulated as follows:

$$\text{Yeast yield in \%} = \frac{100 \times \text{g. newly formed dry yeast substance}}{\text{g. sugar consumed in yeast formation}}.$$

It is of great interest to insert in the denominator the values found by various authors (*cf.* Sperber, 1945) for the synthesis of the elementary components of the yeast. If we assume that 2.0 g. of glucose is consumed in the formation of 1 g. of yeast protein or carbohydrate, and  $y$  g. of glucose in that of 1 g. of fat, we obtain the following equation:

$$\text{Yeast yield in \%} = \frac{100 \times \text{g. newly formed yeast}}{2.0 (\text{g. protein} + \text{g. carbohydrate}) + y \times \text{g. fat}}.$$

From four experiments which appear to have given the optimum result, the following data were obtained:

TABLE VI  
*Quantity of Sugar Consumed/g. of Newly Formed Fat*

Experiment	Yeast yield	Protein + carbohydrate	Fat	Ash	g. glucose consumed/g. fat = $y$
		<i>per cent</i>			
I	28.1	31.9	64.8	3.3	4.51
II	29.1	36.1	59.4	3.5	4.57
III	31.4	42.6	53.8	3.6	4.34
IV	40.5	73.0	21.2	5.8	4.77
<i>T. utilis</i>	52.5	89.0	3.0	8.0	4.18

Mean value for experiments I-IV

4.55

The consumption of glucose in the synthesis of the fat and carbohydrate of the yeast is thus approximately inversely proportional to the energy contents of these substances.

*f. Cultivation Experiments with a Generous Supply of Nutrient Salts for Obtaining Rapid Growth of Yeast*

In view of the fact that the growth rate of the yeast was low during the period of fat formation, it was of special interest to study the growth under conditions which should especially favor it.

A cultivation under such conditions was carried out in the following manner:

The apparatus was provided with 30 l. of a substrate having the following composition, the temperature being 27–29° C. and the culture time 82 hours:

	g./l.
Invert sugar	60.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	15.0
K <sub>2</sub> SO <sub>4</sub>	3.0
KH <sub>2</sub> PO <sub>4</sub>	4.7
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	3.0
NaCl	1.5
CaCl <sub>2</sub> ·6 H <sub>2</sub> O	1.5
FeCl <sub>3</sub>	0.015
+ 60 ml. 20% beer wort.	

During a part of the incubation period (26<sup>h</sup>–33.50<sup>h</sup>) continuous determinations were made of the yeast yield and the generation time (see Table VII).

TABLE VII  
*Generation Time in Growth Period of Pure Cultivation*

Time in hours	Sugar	Dry yeast substance	Yeast yield	Generation time (hrs.)
	g./l.	g./l.	per cent	
26	48.7	5.24		
31.25	17.9	18.9		2.8
32.25	7.40	24.9		2.8
33.50	4.50	27.5	50.4	3.1

These values show that *Rh. gracilis* is comparable, under favorable conditions, with *T. utilis*, both as to yield and generation time of the yeast. Under such conditions, however, very little fat is formed. Fat determinations were not made in this experiment. Later in the cultivation (42<sup>h</sup>), when the nitrogen-content of the substrate had decreased, the fat content was only 9.35%. The generation time had then risen to ca. 6 hours. It is probable that after the above-mentioned rapid

growth the yeast did not contain any more fat than compressed yeast or *T. utilis* would under similar conditions.

*g. Continuous Cultivation with High Fat Formation by the Yeast*

As fat yeast with a high fat content has a low rate of growth, while fodder yeast (*T. utilis*) during continuous cultivation is in a state of the greatest reproductive vigor, it may be presumed that the continuous cultivation of fat yeast of high fat content cannot compare favorably, in the economic sense, with that of fodder yeast, in view of the long generation time of the former.

A preliminary experiment with practically continuous introduction of nutrient solution and tapping-off of the yeast gave a result in full concordance with the above reasoning. The yeast yield and the fat coefficient were in agreement with our experiences in the discontinuous experiments, and the cultivation appeared to have proceeded normally in this sense. The generation time, 51 hours, was, however, quite irreconcilable with the requirements of industrial practice. Even if this single experiment, performed over a short time, cannot be taken as providing strong evidence, it nevertheless supports the supposition that wholly continuous cultivation of *gracilis* yeast with high fat content is very unfavorable from the industrial point of view.

As has been pointed out on page 385, a semi-continuous process under certain conditions may be worthy of consideration from the technical point of view.

### SUMMARY

(1) By drastic reductions in the supply of N and P during aerated cultivation of *Rhodotorula gracilis* the fat content of the yeast could be considerably increased. The minimum value for the N concentration in the substrate 0.6 g. N/100 g. glucose appears to be.

(2) In non-continuous cultivation on the 50-l. scale, fat contents of between 50 and 60% were obtained, the *fat coefficient* (Rippel) being 16-18. The generation time was 15-20 hours and the protein content amounted to only 12-13%.

(3) It is disadvantageous as regards sugar economy to extend the formation of fat over a long period as the fat coefficient gradually decreases.

(4) For the formation of 1 g. fat in *gracilis*  $\pm$  4.5 g. glucose is required.

(5) When the supply of N and P was plentiful very short generation times were obtained (as low as 2.8 hours), but the fat contents in these cases were low.

(6) Continuous cultivation of *gracilis* with limited supplies of N and P proved to be unfavorable, as the generation time rose to 50-60 hours.

The expenses of this investigation have been borne by the Academy of Engineering Sciences, Stockholm. For this we wish to extend our sincere thanks. We express our gratitude to Prof. Edy Velander, the Director of the Academy, for the great personal interest he has taken in the investigation.

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# The Inhibition of Bacterial Growth by Dibromosalicil\*

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## INTRODUCTION

Kuhn, Birkofer and Moller (1) described the antibiotic effects of five benzil derivatives in 1943 and reported that 5,5'-dibromo-2,2'-dihydroxybenzil (dibromosalicil) was a particularly powerful inhibitor for *Staphylococcus aureus*.

A study of dibromosalicil was nearing completion in this laboratory when further information became available through reports released by the War and Navy Departments (2). According to these reports, which were based on interviews with Dr. Kuhn, clinical trials with dibromosalicil had been started in Germany but were interrupted at the time of the occupation. The reports gave an account of the clinical results obtained up to that time and made dibromosalicil appear to be an extremely promising chemotherapeutic agent. It was stated that the compound had been used in the form of a dusting powder on a number of patients with infected war wounds and was found to be as effective as penicillin. Chronic gonorrhea in women had been treated by vaginal insufflation of a dusting powder, apparently with excellent results. It was furthermore claimed that the oral administration of not more than 20 mg. dibromosalicil/day cleared up cases of sulfonamide- and penicillin-resistant gonorrhea within three days. Finally, a patient with a *Staph. aureus* septicemia received the material intravenously for six days with, according to the interview, "phenomenal" results, but further treatment was prevented by military operations.

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The experiments to be described here confirmed the fact that dibromosalicil is a potent antibacterial agent of low toxicity. However, from *in vitro* experiments as well as from clinical studies, we have arrived at the conclusion that dibromosalicil has only a very slight antibacterial effect in presence of plasma and is, therefore, of little value for the treatment of systemic infections.

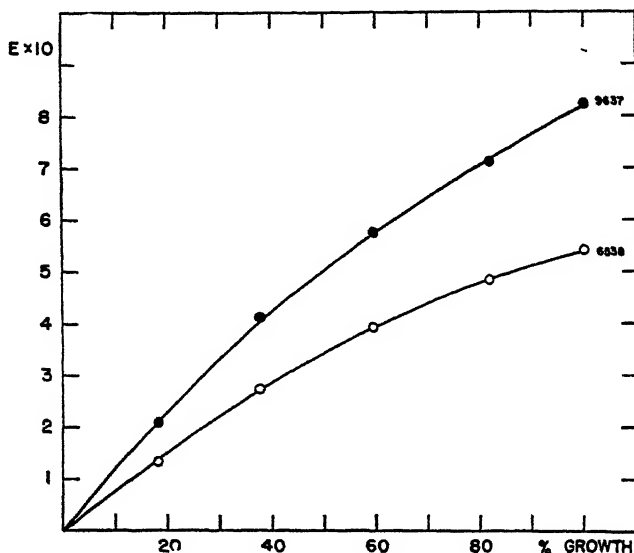


FIG. 1

Calibration Curves Relating Turbidity and Percentage Growth for  
*E. coli* No. 9637 and *Staph. aureus* No. 6538

## EXPERIMENTAL

### 1. Synthesis of 5,5'-Dibromo-2,2'-Dihydroxybenzil (1)

Heating of *o*-methoxybenzaldehyde in 50% alcohol in presence of KCN (3) produced 2,2'-dimethoxybenzoin in a yield of about 50%. Oxidation with Fehling solution (4) gave 2,2'-dimethoxybenzil in a yield of 84%. It was observed that treatment of the dimethyl ether with  $\text{AlCl}_3$  in nitrobenzene solution (1) produced dihydroxybenzil only if the  $\text{AlCl}_3$  was completely dissolved in nitrobenzene before the addition of dimethoxybenzil. From 10 g. dimethoxybenzil usually 5.4–6.2 g. (60–72%) dihydroxybenzil were obtained; occasionally, however, the yields reached only 30–40%. For the bromination, dihydroxybenzil (10 g.) was dissolved in 300 ml. glacial acetic acid and warmed to 50°C. Bromine (13.2 g. dissolved in 100 ml.  $\text{CH}_3\text{COOH}$ )

was slowly added from a dropping funnel. Yellow crystals began to separate from the solution and after all the bromine had been added, the temperature was kept for an additional  $\frac{1}{2}$  hr. at 50–60°C. with frequent mixing. Reflux condenser and dropping funnel were then removed and the temperature was raised and kept at 100°C. for  $\frac{1}{2}$  hr. to boil off HBr and excess bromine. Four hundred ml. water were then added, the mixture was cooled and 5,5'-dibromo-2,2'-dihydroxybenzil was filtered off and dried *in vacuo* over  $\text{CaCl}_2$  and KOH. Yield 15.5 g. (94%); m.p. 206°C., constant after recryst. from  $\text{CH}_3\text{COOH}$ . Kuhn *et al.* found a m.p. of 212–213°C.

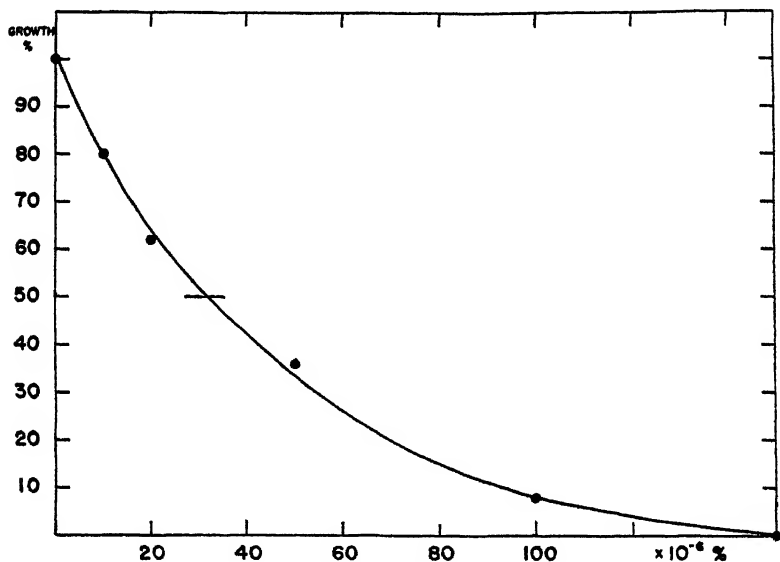


FIG. 2

Inhibitory Effect of Dibromosalicil on the Growth of *Staph. aureus*  
Complete inhibition: 0.15 mg./100 ml.; 50% inhibition: 0.032 mg./100 ml.

### 2. Determination of Antibacterial Activity

The organisms used were *Escherichia coli* (A.T.C.C. No. 9637), *Staphylococcus aureus* (A.T.C.C. No. 6538), *Bacillus subtilis* (A.T.C.C. No. 6633), *Bacillus mycoides* (A.T.C.C. No. 6462, resistant to penicillin) and *Streptococcus Lancefield H 69-D 5* (a non-pathogenic group D hemolytic streptococcus). *B. subtilis* and *B. mycoides* were grown on the agar medium described by Waksman and Reilly (5). For bacteriostatic testing, 10 ml. of agar medium were melted and mixed with 1 ml. of a solution of the test substance. After cooling, one half of the plate was inoculated with *B. subtilis* in three sections, without recharging the platinum loop, and the second half in the same manner with *B. mycoides*. Control plates without test substance were set up for comparison and showed full growth at 25°C. after 32–48 hr. Suspensions

for streaking the plates were obtained by adding 5 ml. of sterile water to a 48 hr. growth on agar slants and scraping with a platinum needle.

Bacteriostatic activity against *Staph. aureus*, *E. coli* and *Strep. Lancefield* was measured turbidimetrically. Fresh cultures (18 hr.) grown in medium II of Schmidt and Moyer (6) were diluted with sterile medium. The dilutions used were  $10^{-6}$  for *E. coli*,  $10^{-5}$  for *Staph. aureus* (which gave a concentration of 2000-3000 organisms/ml.) and  $10^{-3}$  and  $10^{-6}$  for *Strep. Lancefield*. Ten ml. of these diluted cultures were pipetted into sterile Pyrex test tubes (18.5 mm. diameter) containing 1 ml. solution of the test substance in various concentrations. The mixtures were incubated for 18 hr.

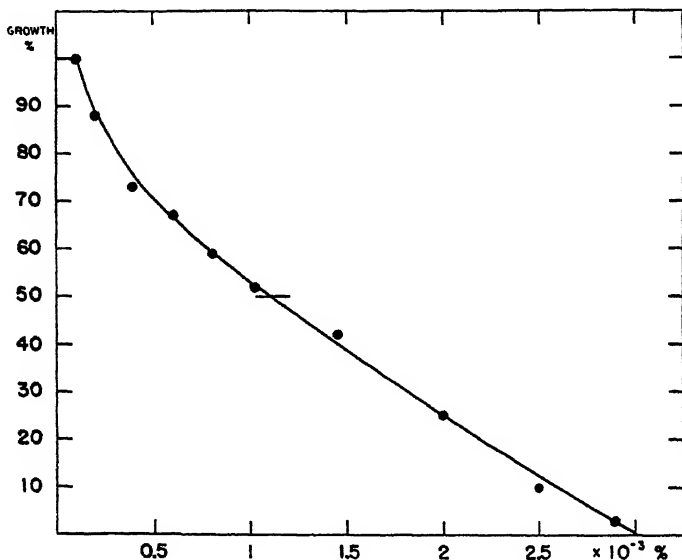


Fig. 3

*Inhibitory Effect of Dibromosalicyl on the Growth of Strep. Lancefield*

Culture dilution:  $10^{-3}$ , complete inhibition by 3 mg./100 ml.; 50% inhibition by 1.1 mg./100 ml. For a culture dilution of  $10^{-6}$  the corresponding concentrations were 1 mg./100 ml. and 0.4 mg./100 ml.

at 37°C. After careful mixing, the turbidity in each tube was then read against sterile medium with a Lumetron Photoelectric Colorimeter 402-E, Filter M-575. The extinction values obtained corresponded to a certain percentage of full growth which was read from calibration curves. Typical calibration curves are shown in Fig. 1. They were prepared by reading the turbidity of various dilutions of 18 hr. cultures with sterile medium and plotting extinction values against dilution or the corresponding percentage of full growth.

The curves shown in Figs. 2-4 illustrate the effect of increasing amounts of dibromosalicyl on the growth of *Staph. aureus*, *Strep. Lancefield* and *E. coli*. The results ob-

tained with *B. mycoides* and *B. subtilis* are summarized in Table I. A few additional organisms isolated from patients of local hospitals were also cultured in presence of dibromosalicil. A strain of *Staph. aureus* from a patient with penicillin- and sulfadru-resistant septicemia was completely inhibited by 0.2 mg. dibromosalicil/100 ml. medium. A strain of *Strep. viridans*, resistant to 50 mg./100 ml. sulfadiazine and to

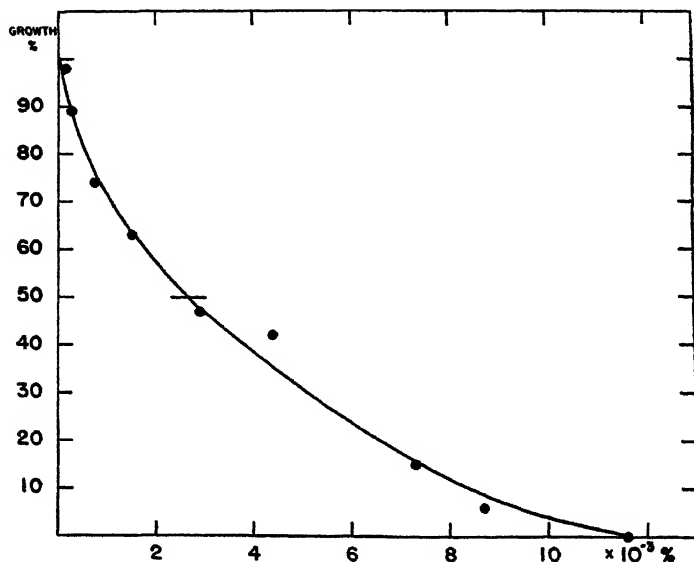


FIG. 4

Inhibitory Effect of Dibromosalicil on the Growth of *E. Coli*  
Complete inhibition: 11.6 mg./100 ml.; 50% inhibition: 2.65 mg./100 ml.

TABLE I

*Effect of Dibromosalicil on the Growth of B. mycoides and B. subtilis*

Dibromosalicil mg./100 ml.	<i>Bacillus mycoides</i>		<i>Bacillus subtilis</i>	
	20 hr.	70 hr.	20 hr.	70 hr.
0.20	—	—	—	—
0.10	+	+	+	+
0.05	+	++	+	++
0.02	++	+++	+	+++
zero	++	+++	++	+++

The absence of visible growth is denoted by the symbol —, while + to +++ denote increasing amount of growth.

5 units penicillin/ml. failed to grow at a dibromosalicil level of 1 mg./100 ml. The same concentration inhibited *Pneumococcus*, whereas 4 mg./100 ml. were required to completely inhibit a strain of  $\alpha$ -hemolytic streptococcus.

### 3. Toxicity

Dibromosalicil, in form of its borate complex (1), injected intravenously into white rats was well tolerated in amounts up to 200 mg./kg. body weight. Oral administration of dibromosalicil in amounts up to 200 mg./day for one week produced no symptoms in human volunteers. Intravenous administration of the borate complex to humans in amounts up to 500 mg./day for 6 days was also well tolerated.

### 4. Bactericidal Tests

Mixtures of 0.5 ml. human blood (drawn under sterile precautions and defibrinated by mixing with glass beads), 0.05 ml. dibromosalicil solutions, and 1 drop diluted *Staph. aureus* cultures were sealed in glass tubes and rotated mechanically 6 times/hr. for 48 hr. at 37°C. The final dibromosalicil concentration in the various tubes ranged from 0.1 to 40 mg./100 ml.; the number of organisms introduced varied from 1 to 1 million, with a ten-fold increase in number from one tube to the next. At the end of 48 hr. the tubes were opened and dilutions were plated out for bacterial counts. Even tubes which had been seeded with only ten organisms and contained 40 mg. dibromosalicil/100 ml. were found now to have a bacterial population of several hundred thousand. The conclusion was reached, therefore, that dibromosalicil in presence of blood did not prevent the growth of *Staph. aureus*.

### 5. Effect of Plasma and Albumin on the Antibacterial Activity of Dibromosalicil

Preliminary experiments indicated that the antagonistic effect of blood on the antibacterial activity of dibromosalicil was associated with a plasma constituent. Whereas 0.15 mg. dibromosalicil/100 ml. medium completely inhibited the growth of *Staph. aureus*, 4 mg./100 ml. were required to prevent visible growth if the medium contained 5% human plasma. Quantitative experiments with bovine albumin (fraction V) showed that the action of dibromosalicil is apparently inhibited by combination of the substance with albumin. Solutions of albumin in 2% NaCl were sterilized by Seitz filtration and added to medium II so that final albumin concentrations of 0.5% and 1% resulted. Calibration curves and curves representing growth in presence of dibromosalicil were obtained for these albumin-containing media as described above for the original medium II. *Staph. aureus* A.T.C.C. No. 6538 was used as test organism. The results were as follows:

Albumin g./100 ml.	Dibromosalicil mg./100 ml. required for 50% inhibition
0	0.032
0.5	3.3
1.0	4.9

### 6. Clinical Experiments

The following clinical studies were carried out by Dr. Thomas Findley and Dr. Mortimer Silvey. One patient with subacute bacterial endocarditis caused by a

penicillin- and sulfa drug-resistant strain of *Strep. viridans* was given 300–600 mg. dibromosalicil (as borate complex) intravenously daily for one week. Positive blood cultures were obtained at the end of this period. *In vitro*, the organism isolated from the blood of this patient failed to grow when a dibromosalicil level of 1 mg./100 ml. was reached. Seven male patients with gonorrhea did not respond to the oral administration of 20–65 mg. dibromosalicil/day for 6 days.

## DISCUSSION

Kuhn *et al.* (1) determined the concentration of dibromosalicil required for complete inhibition of *Staph. aureus* as 0.1–0.2 mg./100 ml., which is in good agreement with our value of 0.15 mg./100 ml. Our investigation of the effect of this substance on the growth of other bacteria revealed good activity against a variety of microorganisms. It is particularly noteworthy that *E. coli*, a representative of the gram negative group, was inhibited too, although the effective dibromosalicil concentrations were considerably higher than those required against gram positive organisms.

Our failure to achieve a cure of gonorrhea and *Strep. viridans* septicemia with dibromosalicil is understandable in view of the marked decrease in activity of the substance in presence of plasma and albumin. With an albumin concentration of 1%, which is only about  $\frac{1}{4}$  of that in normal plasma, the dibromosalicil level had to be increased about 150-fold to prevent growth by 50%, as compared to the level effective in medium II. Our bactericidal tests showed furthermore, that *Staph. aureus*, when growing in human blood, is not inhibited even at a dibromosalicil concentration of 40 mg./100 ml., which is about 270 times the concentration necessary for complete suppression of the same organism in medium II and 1250 times the concentration required for 50% inhibition. It might be added here that dibromosalicil was not antagonized by *p*-aminobenzoic acid in concentrations up to 1 mg./100 ml., in contrast to the sulfa drug group.

No explanation can be offered for the claims (2) based on preliminary clinical trials in Germany. It is certain, however, that the quantities used in these clinical experiments were not sufficient to overcome the antagonistic effect of albumin. In absence of plasma, dibromosalicil is a powerful bacteriostatic agent and it may be useful for the treatment of local infections and for the protection and preservation of a variety of products.



## SUMMARY

1. The inhibitory action of 5,5'-dibromo-2,2'-dihydroxybenzil on the growth of a variety of bacteria was determined quantitatively.

2. The powerful antibacterial activity of dibromosalicil was found to be markedly reduced in presence of blood, plasma or albumin.

3. Dibromosalicil was found to be relatively non-toxic. White rats tolerated intravenous administrations in amounts up to 200 mg./kg. without toxic symptoms.

4. Oral administration of dibromosalicil was of no benefit to 7 patients with gonorrhea. The condition of a patient suffering from subacute bacterial endocarditis was not improved by intravenous treatment.

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# The Nitrogenous Constituents of Flaxseed. IV. The Isolation of Purified Conlinin (with a Note on an Improved Linin Isolation Procedure)<sup>1</sup>

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## INTRODUCTION

The isolation and partial characterization of the individual proteins of flaxseed were initiated in paper II of this series (1). Under our conditions of nitrogen extraction and protein precipitation, the first crude protein precipitate contained a mixture of several proteins. One of these, named linin, was isolated as a homogeneous entity and some of its chemical and physical properties were described. Its purification depended largely on the separation of a second protein which was named conlinin. It is the purpose of this communication to describe the isolation of what appears to be homogeneous conlinin, and to record some of its chemical and physical constants. A modified isolation procedure for linin will also be reported; it is a simplification of the previously described method, and yields somewhat larger amounts of the protein.

The previously described separation procedure depended upon the precipitation of linin from an alkaline solution of the 2 proteins by HCl at pH 5.7, at which pH pure conlinin was found to be soluble. The latter was then precipitated at pH 4.5. Conlinin, however, showed a strong tendency to partially coprecipitate with linin at pH 5.5-5.7, which necessitated frequent redispersions and precipitations. While searching for a better method of separation it was observed that linin would precipitate readily with CO<sub>2</sub>, and carry down less conlinin than when precipitated with HCl or CH<sub>3</sub>COOH.

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<sup>1</sup> Published by permission of the Director, North Dakota Agricultural Experiment Station. This work was carried out under Purnell Project 95, "The Chemistry of Flaxseed."

The solubility of pure linin in water or in acidic solutions of 5% sodium chloride, sodium sulfate and ammonium sulfate is low, but at pH 8.0, or above, pure linin dissolves rapidly. However, to form stable dispersions of crude linin, higher pH values are required. We, therefore, adopted pH 10.2 as a convenient alkalinity, one at which crude linin would dissolve and one at which, according to available evidence, no denaturation occurred. During the course of this investigation it appeared that conlinin was not as alkali-stable as linin, for if partially purified conlinin was repeatedly dissolved at pH 10.2 and the pH lowered stepwise to 5.5 and 4.5, respectively, less and less conlinin could be recovered after each new cycle. It appeared, therefore, necessary for the purpose of this investigation to find the minimum alkalinity at which crude linin would dissolve without causing appreciable conlinin losses. Tentatively, until more exact data have been gathered, this was set at pH 9.0, and the previously described method was modified accordingly.

### EXPERIMENTAL

The extraction of the ground, lipid-free linseed meal with phosphate-buffered ethylene glycol at pH 7.2 has been described (1). After centrifugation and filtration of the supernatant liquid through cheesecloth, the pH is adjusted to 9.0 with saturated  $\text{Na}_2\text{CO}_3$ . One volume of water is added, followed by the slow addition of one volume of dioxane to the mechanically stirred solution maintained at pH 9.0. The dioxane-insoluble gummy material settles more slowly at pH 9.0 than previously at pH 10.2, but within 24 hours it flocculates as completely and centrifuges as well as formerly. The clear yellow solution is filtered, diluted with 1.5 volumes of water, and acidified with concentrated HCl to pH 7.0. Ammonium sulfate is added to give a 5% salt concentration, and the pH is lowered to 4.5–4.6 with concentrated HCl. The proteins settle readily, leaving a faintly opalescent, light yellow supernatant solution which can be siphoned off. The residue centrifuges well. In the previously published method no salt addition was recommended. It is now, however, because while work was being done on the isolation of the globulins and albumins by salt fractionation of the supernatant, it became apparent that appreciable amounts of linin and some conlinin had escaped the original precipitation, even though the mother liquor from the pH 4.5 protein precipitation had been essentially free of turbidity.

To the supernatant solution 5 g. of ammonium sulfate/100 cc. of solution are added and the pH adjusted to 3.2. A further protein precipitate forms which does not settle well but which centrifuges readily. The proteins precipitating at pH 3.2 are a mixture of a little linin, some conlinin and some other proteins. Since the volume from which the pH 3.2-insoluble precipitate was obtained is large, and since the total amount of proteins precipitating at pH 3.2 is relatively small and requires centrifugation, the working up of this fraction is time consuming and is not ordinarily recommended.

However, it has been done in the case of the 3 preparations mentioned below where yield data are given.

The proteins from the combined precipitates at pH 4.5 and 3.2 from each 250 g. of original meal are dissolved with mechanical stirring in 10 l. of water adjusted to pH 9.0 with saturated  $\text{Na}_2\text{CO}_3$  solution. Because the crude linin dissolves slowly the solution should be stirred for about 18 hours, and the pH, which tends to drop, should be frequently readjusted to 9.0. The solution remains extremely opalescent, and should not be filtered to avoid low protein yields. Carbon dioxide is now passed into the rapidly stirred solution until the pH is 5.4–5.5. Foaming is controlled by an occasional addition of a few drops of capryl alcohol. A heavy white precipitate settles rapidly. The precipitated proteins are dissolved several times in 10 l. of water at pH 9.0, and reprecipitated with  $\text{CO}_2$  until a supernatant is free of conlinin. This generally occurs after the 4th or 5th reprecipitation, and is ascertained as follows: to 500 cc. of an aliquot of the supernatant 34 g. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  is added and the pH carefully adjusted to a permanent 5.5 value with 25%  $\text{CH}_3\text{COOH}$ . After 12 hours, the solution is filtered and  $\text{CH}_3\text{COOH}$  is added to pH 4.55–4.60. If no precipitate forms on standing, the separation of conlinin can be considered essentially complete.

#### *Purification of Linin*

The last  $\text{CO}_2$  precipitate of linin is dissolved in 1000 cc. of NaOH at pH 10.2 and filtered. A slightly opalescent filtrate is obtained from which linin is precipitated with  $\text{CO}_2$ . The supernatant is tested for conlinin and, if free from it, is discarded. The protein is once more dissolved in 250 cc. of NaOH at pH 10.2, filtered, diluted to 1000 cc. and precipitated with HCl at pH 5.5–5.6. At this state of purity, yield data are available for 3 preparations from 250 g. of meal each: 6.6, 7.1 and 6.1 g. of linin calculated/100 g. of meal, corrected for moisture and ash. The nitrogen content of the last preparation was 17.41% (corrected). The first 2 preparations were combined with the product of a large scale extraction of linseed meal from which more than 100 g. of linin were obtained. The analytical values for this latter preparation are: moisture 6.0%, ash 0.2%, nitrogen<sup>2</sup> (corrected) 17.32%, sulfur (corrected) 0.65%. The nitrogen and sulfur values are slightly higher than the 17.0% and 0.60% of the homogeneous preparations previously described (1, Table VIII). It is evident that the  $\text{CO}_2$  precipitated protein requires a further purification with formic acid, or by equilibrating with buffer at pH 5.7 (1). Part of the preparation was, therefore, treated with formic acid. The nitrogen and sulfur values (corrected) changed to 17.05%<sup>3</sup> and 0.61%. These values now agree with those previously reported for highly purified linin. From a 10 g. sample of  $\text{CO}_2$ -treated linin, 8.1 g. were recovered after formic acid treatment.

#### *Purification of Conlinin*

The first 4 supernatants from the  $\text{CO}_2$  precipitations of linin are combined, 68 g. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  are added/1000 cc. of solution, and the pH is carefully adjusted to 5.45 with  $\text{CH}_3\text{COOH}$ , care being taken that the pH does not go below 5.4 at any time. Appreciable amounts of linin precipitate and are centrifuged, dissolved in 1000 cc. of  $\text{Na}_2\text{CO}_3$  solution at pH 9.0 and precipitated with  $\text{CO}_2$ . This linin precipitate is included, after further purification, in the yield data previously mentioned. To the supernatant are added 68 g. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ /1000 cc., the pH is adjusted to 5.45

and any unprecipitated linin is filtered off. The filtrate is combined with the bulk of the conlinin solution. To it are added 5 g. of  $(\text{NH}_4)_2\text{SO}_4$ /100 cc. of solution, and the pH is adjusted to 4.55 with glacial  $\text{CH}_3\text{COOH}$ . The conlinin settles and centrifuges well, but the supernatant, even though free of turbidity, contains small amounts of unprecipitated conlinin, probably due to the high dilutions. Since the yield of conlinin, compared with that of linin, is small, we have made it a practice to recover the unprecipitated conlinin by increasing the  $(\text{NH}_4)_2\text{SO}_4$  concentration to 15%. The residual conlinin flocculates and centrifuges poorly at the density of the 15% salt solution. However, it filters rapidly. The filtrate is kept for the isolation of additional flax proteins which will be described at a future date. The conlinin is dissolved from the filter paper in 500 cc. of  $\text{Na}_2\text{CO}_3$  at pH 9.0, and the filtered solution used to dissolve the bulk of the centrifuged conlinin. After filtration, 68 g. of  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ /1000 cc. are added, the pH adjusted carefully with acetic acid to 5.45 and, after 12 hours' standing the solution is filtered. To the filtrate are added 30 g. of  $\text{NaCl}$ /1000 cc. solution, and conlinin is precipitated at pH 4.55. The added  $\text{NaCl}$

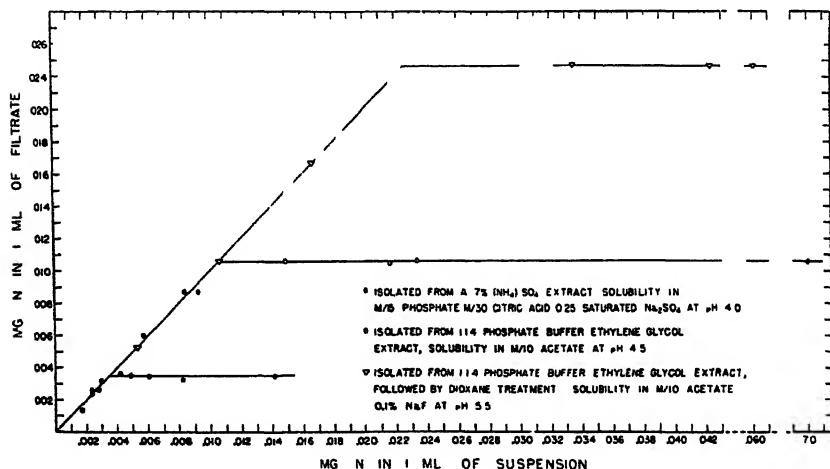


FIG. 1

Solubilities of 3 Conlinin Preparations at 25°C. in Different Salt Solutions and at different pH Values, in the Presence of Increasing Quantities of Solid Phase<sup>2,3</sup>

<sup>2</sup> The nitrogen analyses from which the upper curve was drawn, and those shown in Table I, were performed by Mr. George G. Maher, for which grateful acknowledgement is made.

<sup>3</sup> The nitrogen analyses from which the lower two curves were drawn, and those shown in Table I, were performed by Mr. L. L. Nesbitt, for which grateful acknowledgement is made (present address: Limestone Products Corporation of America, Newton, N. J.).

decreases conlinin's solubility without causing precipitation of some of the other flaxseed protein fractions which require higher salt concentrations and lower pH values. This process of solution at pH 9.0 and fractional precipitation at pH 5.45 and 4.55 is repeated, usually 4-5 times, until 2 successive treatments yield solutions at pH 5.4 which are free of opalescence but show the first signs of turbidity at pH 5.1, and which are free of proteins at 20% NaCl concentration, pH 3.2, after the removal of the conlinin.

The three 250 g. meal extractions for which yield data were previously given for linin, were also used to get yield data on conlinin. We isolated 0.4, 0.5 and 0.3 g. of conlinin calculated/100 g. of meal, and corrected for moisture and ash. Because of the small amounts of available conlinin, these 3 preparations were combined and the data presented in Fig. 1 and in the last column of Table I are those of the combined preparations. Data are also presented in Fig. 1 and Table I, for 2 other conlinin preparations, one of which was the first conlinin we obtained from a 5%  $(\text{NH}_4)_2\text{SO}_4$  extraction of the meal, and a second obtained from phosphate-buffered ethylene glycol extracts from which the gummy impurities were removed by repeated filtrations instead of by dioxane precipitation.

TABLE I  
*Chemical and Physical Data on Three Conlinin Preparations\**

	Conlinin prepared from:		
	$(\text{NH}_4)_2\text{SO}_4$ - extracted meal	Phosphate-buffered ethylene- glycol-extracted meal	
		Filtered but not dioxane treated	Dioxane treated
Nitrogen %	16.86 <sup>a</sup>	17.03 <sup>a</sup>	16.72 <sup>a</sup>
Sulfur %	0.79	0.72	0.76
Carbohydrate %	0.83	0.44	1.48
Isoelectric point (from Fig. 2)			4.60
Approximate solubility in water brought to the indicated pH from acid or alkaline solutions			below 3.9 above 5.1

\* All values are calculated on ash- and moisture-free basis.

Homogeneity of each preparation was established by the application of the phase rule to solubility data in accordance with Butler's (2) modification of the Northrop and Kunitz (3) method. The solubility of each of the 3 preparations shown in Fig. 1 was determined at a different pH, and in different salt solutions from that of the others, yet in each case the slope of the not fully saturated solution was 1,

and the solubility at full saturation was independent of the amounts of undissolved conlinin. It is, therefore, quite evident that all 3 preparations were homogeneous as far as protein constituents are concerned.

Table I gives the percentage of nitrogen, sulfur, phosphorus and carbohydrate in each preparation. (For methods see (1).) The nitrogen contents of the 3 preparations were 16.72, 16.86 and 17.03%. These differences are not due to moisture or ash, for corrections have been applied, unless the ash contained substances which were volatile at

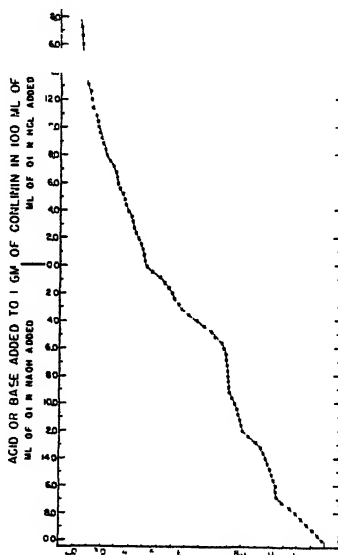


FIG. 2

Electrometric Titration Curve of Conlinin between pH 2.0 and 11.0

600°C. However, the carbohydrate content of the highest nitrogen-containing preparation was only 0.44%, compared to 0.83 and 1.48% for the 2 other preparations. If the latter 2 are corrected to a 0.44% carbohydrate value, the calculated nitrogen content of these proteins would be 16.90% and 16.93%, hence, more like that of the 17.03% protein. The sulfur values show better agreement, namely, 0.79, 0.72 and 0.76%. No phosphorus was found in any of these preparations.

To more closely approximate the isoelectric point, which was assumed to be close to pH 4.5 from the insolubility of conlinin at this pH, an electrometric titration was performed as previously described (1). According to the titration, the isoelectric point is at or near pH 4.60. The curve shows a number of strongly absorbing regions, differing considerably in this respect from the titration curve of linin (1, Fig. 2).

#### *Linin Precipitation at pH 0.5*

An interesting nitrogen dispersion curve of oil-free linseed meal was recently published by Smith, Johnsen and Beckel (4). The graph showed 2 solubility maxima, at pH 1.0–1.2 and in the alkaline range, as well as 2 minima, at pH 0.5–0.6 and 3.5–4.5. Of particular interest to us was the maximum at pH 1.0–1.2 and the minimum at pH 0.5–0.6 because their data indicated that the bulk of the flaxseed proteins showed this characteristic behavior. Hitherto we had tested the solubility of linin at pH values of 2 or above but not in the more acid ranges. Consequently, a 5 times CO<sub>2</sub>-precipitated linin preparation from 250 g. of linseed meal was suspended in 500 cc. of distilled water, then acidified with HCl to pH 3.0 where all of the protein dissolved, giving a yellow, somewhat turbid solution. Upon the dropwise addition of concentrated HCl to the mechanically stirred solution, increased opalescence was observed from pH 1.8 down to pH 1.0 but 24 hours' standing at the latter pH caused no protein precipitation. The pH was further decreased; at pH 0.7 the first definite precipitation occurred but was incomplete; at pH 0.5 linin settled rapidly, leaving a slightly yellow, clear supernatant. After centrifugation the protein was suspended in 500 cc. of distilled water and the pH adjusted to 3.0 with 50% NaOH. Very little protein dissolved during 24 hours, but at pH 9.0 solution occurred. The entire cycle was repeated once more without a noticeable alteration in the solubility of linin. It was suspected that the reason why linin, precipitated at pH 0.5, could not be dissolved directly by pH adjustment to 3.0 was the accumulation during neutralization of enough NaCl to suppress the already low solubility of linin in acid solution. Consequently, freshly CO<sub>2</sub>-precipitated protein was suspended in 1% NaCl solution at pH 3.0 and stirred for 24 hours. The major part of the protein remained undissolved and, only after stepwise dilutions to a salt concentration of 0.2%, was solution effected. It was stable, although very turbid.



A small sample of purified conlinin in distilled water suspension dissolved rapidly at pH 3.0. At pH 0.5 it exhibited considerable opalescence but no flocculation could be observed during the following 24 hours. Neutralization to pH 4.55 precipitated the protein. It is possible that the differences in solubility of linin and conlinin at pH 0.5 are sufficient to permit their separation in acid solutions, provided that conlinin does not show the same tendency to coprecipitate as it does at pH 5.5–5.7. Such an isolation procedure would have the advantage of not exposing conlinin to alkali.

### DISCUSSION

Osborne (5) described a number of flaxseed protein fractions. One of these possesses some properties similar to some of conlinin's, although other properties are dissimilar. His water extracts of linseed meal yielded solutions with an acid reaction from which, on the addition of weak  $\text{CH}_3\text{COOH}$ , a protein precipitated which redissolved in an excess of the acid. The sulfur contents of 2 such preparations were 0.73 and 0.76%. Conlinin resembles Osborne's protein in solubility to the extent that it too is soluble in weak acid solutions (pH 5.1–6.9), is precipitated on the addition of weak  $\text{CH}_3\text{COOH}$  (pH 4.5–4.6), and redissolves in an excess of the acid (below pH 3.9). The sulfur values are also similar, 0.72–0.79%. However, Osborne's protein contained 18.61–18.74% nitrogen and was crystalline, while conlinin's sulfur content was 16.72–17.04%, and all preparations were amorphous.

In this investigation, as in the case of the previous one (1), main emphasis was placed on homogeneity of product rather than on yield. Since, however, yield is at times of importance, attention should be called to several recent publications which suggest a number of modifications of this method, with consequent higher protein recoveries. Painter and Nesbitt (6) reported that 6 hours ball-mill grinding of roller-mill ground linseed meal increased the amounts of nitrogen extractable with 1 *N* NaCl from 58.8 to 79.0%. More recently<sup>4</sup> they extracted ball-mill ground meal with phosphate-buffered ethylene glycol and recovered 78–80% of the meal nitrogen, which is about 20% more than we were able to extract from roller-mill ground flaxseed meal (1). Smith, Johnsen, and Becks (4) showed that, by increasing

<sup>4</sup> Grateful acknowledgment is made to E. P. Painter and L. L. Nesbitt for permission to quote their unpublished data.

the meal:solvent ratio of a decorticated, hexane-extracted linseed meal from 1:10 to a 1:50 ratio, the first extraction yielded 74.9% recoverable nitrogen instead of 48.8% at the lower ratio. The corresponding values for 2 successive extractions were 87.2% against 75.5%. It was also shown previously (1) that a third extraction with buffered ethylene glycol peptized an additional 6.3% of meal nitrogen. We have, however, not used a third extraction because the increase in volumes with which one has to work is disproportionately large for the amounts of extra nitrogen one obtains. Finally, still another factor appears to affect yields. Painter and Nesbitt quoted unpublished data<sup>4</sup> in which they found significant differences in the dispersions and precipitations of proteins from different varieties of flax. It should be quite evident from the aforesaid that yields depend in large measure upon the smallest detail of the method used as well as on the flax varieties chosen.

#### SUMMARY

A detailed description of a method suitable for the isolation of homogeneous conlinin preparations from linseed meal is presented. An improvement on the previously published linin isolation procedure is also described.

Three conlinin preparations were isolated which, according to solubility data, were free from contamination by other proteins. Calculated on an ash- and moisture-free basis they were found to contain: 16.72–17.03% nitrogen, 0.72–0.79% sulfur, 0.44–1.48% carbohydrate, less than 0.01% phosphorus, and had, according to electrometric titration and from maximum insolubility data, an isoelectric point of 4.55–4.60.

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# A Colorimetric Method for the Estimation of the Activity of Substances Inhibiting the Isoagglutination of Blood Group A Cells<sup>1</sup>

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## INTRODUCTION

Investigations on the isolation and characterization of blood group specific substances have relied exclusively on serological tests for the determination of the activities of fractions obtained by various procedures. Isoagglutination inhibition and hemolysis inhibition tests have been used and, more recently, a precipitin test has been developed by Kabat and Bezer (1). In the course of work on the isolation of blood group substances from various sources it was considered advantageous to study a color test which appeared suitable for the estimation of the approximate activity of these preparations. This test has proved useful in our work and is of interest since it emphasizes an intrinsic feature of the structure of A-substance.<sup>2</sup>

The test involves the reaction of Ehrlich's *p*-dimethylaminobenzaldehyde reagent with substances which have been previously treated with a hot alkaline solution; the procedure is essentially that described by Morgan and Elson (2) for the estimation of N-acetylglucosamine. This procedure has been found to give a characteristic red-purple color for A-substance isolated from hog gastric mucin (3) and from human

\* Contribution No. 1005.

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology.

<sup>2</sup> In this paper A-substance refers to material which is capable of inhibiting the isoagglutination of human blood group A cells by serum from individuals of blood group B; evidence is available (5) that some materials which are active in isohemolysis tests may have differing properties.

ovarian cysts (4). The reaction appears to possess some specificity since colors are not given by other polysaccharides containing hexosamine such as chondroitin sulfuric acid and hyaluronic acid (3), or by tryptophan (2), which is known to react with Ehrlich's reagent under other conditions (6).

It has been possible, by means of this reaction, to correlate the serological activities of preparations of A-substance, determined by isoagglutination inhibition tests, with the intensities of colors produced with Ehrlich's reagent. The correlation has been applied to fractions obtained from hog gastric mucin by procedures using mild conditions. The empirical relationship derived between serological activity and color intensity then provides a method for the assessment of the activity of subsequent undegraded A-substance preparations simply from the results of the color test.

While the color test possesses the advantages of considerable convenience and speed and somewhat greater precision than the isoagglutination inhibition test, it is emphasized that the method is not general and must be applied with caution to fractions obtained by procedures involving degradation of A-substance. Furthermore, no attempt has been made to investigate exhaustively all factors influencing the color test, since the limited knowledge of the structure of A-substance inevitably restricts investigations of this kind at present.

## EXPERIMENTAL

### *Color Test*

*p*-Dimethylaminobenzaldehyde (Ehrlich's) Reagent. The reagent was prepared by dissolving 2 g. of Eastman White Label *p*-dimethylaminobenzaldehyde in a solution containing 100 ml. of glacial acetic acid and 2 ml. of 12 *N* HCl. The reagent was stored in bottles protected from light.

*Test Solutions of Fractions.* Solutions were prepared by dissolving weighed samples in volumetric flasks and diluting with 0.9% NaCl; the test material concentration was usually 1000  $\gamma$ /ml. It was convenient to use saline as the same solutions could then be used for the serological tests.

*N*-Acetylglucosamine Solutions. Weighed amounts of dry *N*-acetylglucosamine were dissolved in 0.9% NaCl, approximately 0.1 saturated with chloroform. Dilute solutions with concentrations in the range 10–100  $\gamma$ /ml. were prepared by diluting aliquots of more concentrated solutions. It was found that sodium chloride had no significant effect on the color produced with Ehrlich's reagent. *N*-Acetylglucosamine was prepared essentially according to the procedure of Breuer (7) from *d*-glucosamine hydrochloride. The hydrochloride was neutralized with sodium hydroxide before acetylation.

*Procedure for the Analysis of A-substance.* One ml. of A-substance solution<sup>3</sup> and 0.1 ml. of 0.25 *M* Na<sub>2</sub>CO<sub>3</sub> were added to a 15 ml. calibrated centrifuge tube and the solutions mixed. Two controls were prepared: (I) a blank on the reagents (1 ml. of 0.9% NaCl and 0.1 ml. of 0.25 *M* Na<sub>2</sub>CO<sub>3</sub>) and (II) a blank on A-substance (1 ml. of A-substance solution and 0.1 ml. of 0.25 *M* Na<sub>2</sub>CO<sub>3</sub>). The second control was required as A-substance solutions gave a light yellow color after digestion and some solutions gave slight turbidities on final dilutions with acetic acid.

The solutions were heated in a boiling water bath for 15 minutes and cooled by immersion in tap water. The solutions were diluted to 9 ml. with glacial acetic acid and 1 ml. of the *p*-dimethylaminobenzaldehyde reagent added to the A-substance solution and to Control (I). One ml. of glacial acetic acid, 0.24 *N* in HCl (prepared by mixing 1 ml. of 12 *N* HCl and 50 ml. of glacial acetic acid), was added to Control (II). The colors were allowed to develop in a water thermostat at 25.0 ± 0.1°C. for 50–60 minutes. The color intensities were then measured with the Klett-Summerson Colorimeter using a green filter (Klett No. 54); the instrument was preliminarily set at zero against a tube of distilled water. The color intensities were all read in the same Klett tube by rinsing the tube with several portions of solution before adding the major portion. The colorimeter reading for a particular fraction was taken to be equal to the colorimeter reading for the A-substance solution minus the sum of the colorimeter readings for Controls (I) and (II).

*Procedure for the Analysis of N-Acetylglucosamine.* The procedure was the same as for A-substance except that the digestion with alkali was only for 5 minutes, the time recommended by Morgan and Elson (2). Further digestion with alkali produced very rapid decreases in the intensity of the color with Ehrlich's reagent. Control (II) was not necessary as no turbidities are produced with N-acetylglucosamine in the acetic acid solutions.

#### *Confirmatory and Other Experiments*

In the following sections the results of investigations of some of the conditions influencing the color test are summarized.

*Conditions of the Alkaline Digestion.* One-tenth ml. of 0.25 *M* Na<sub>2</sub>CO<sub>3</sub>/ml. of A-substance solution gave a maximum color intensity with Ehrlich's reagent with most preparations after 15 minutes digestion at 100°C. About 60–70% of the maximum color intensity was produced after 5 minutes digestion, 90–95% after 10 minutes digestion and 90–100% after 20 minutes digestion. None of the A-substance samples prepared from hog gastric mucin by fractionation with alcohol (8), sodium sulfate (3) or phenol-alcohol (3), from pepsin by autolysis (8) or alcohol fractionation (9), or by treatment of A-substance with papain-HCN or formamide (8) gave any significant color with *p*-dimethylaminobenzaldehyde without prior alkaline digestion.

For a digestion time of 15 minutes, the concentration of sodium carbonate given above (0.1 ml. of 0.25 *M* Na<sub>2</sub>CO<sub>3</sub>/ml. of A-substance solution) produced the maximum color intensity for an A-substance preparation obtained by sodium sulfate fractionation. The color intensities were 95% and 90% of the maximum color intensity when,

<sup>3</sup> In the present status of the procedure, it is desirable to standardize on a selected concentration of material, e.g., 1000  $\gamma$ /ml. ( $\pm 10\%$ ), due to the non-linear dependence of the color on the concentration of A-substance.

respectively, 0.05 ml. and 0.15 ml. of 0.25  $M$   $Na_2CO_3$ /ml. of A-substance solution were used. The pH of the solution after digestion with 0.1 ml. of 0.25  $M$   $Na_2CO_3$  was 10–10.5; before digestion the pH was usually somewhat higher, 10.3–10.8. If a test solution is highly buffered, or contains acid, the proper pH will not be maintained during digestion and erroneous results will be obtained.

*p*-Dimethylaminobenzaldehyde Reagent. The reagent whose preparation is described above had a pale yellow-orange color which increased in intensity on standing; for this reason the reagent was prepared fresh about every 7–10 days. During the period the reagent was being used, the blank color in the analyzed solutions gave very low colorimeter readings in the Klett-Summerson Colorimeter using a green filter (Klett filter No. 54, approximate spectral range, 500–570  $m\mu$ ). A reagent prepared from *p*-dimethylaminobenzaldehyde recrystallized according to the procedure of Adams and Coleman (10) gave slightly smaller blank colors which increased but little on standing. Furthermore, this reagent, when freshly prepared, gave color intensities for A-substance solutions which were 8–10% greater than those obtained with the unpurified reagent. However, for some reason not thoroughly investigated, this reagent appeared to be somewhat less stable than the reagent prepared from unrecrystallized *p*-dimethylaminobenzaldehyde with respect to the color produced with A-substance; successive analyses of A-substance solutions over a period of several days gave decreasing colorimeter readings. For the purposes of this investigation it was more convenient to use the more reproducible, but somewhat less sensitive reagent prepared from unrecrystallized *p*-dimethylaminobenzaldehyde.

It is apparent from these results that it would be desirable to standardize the procedure so that color intensities produced with A-substance would not reflect changes in the reagent. A few experiments have indicated that N-acetylglucosamine may be a suitable standard as it is stable, can be prepared quite pure, and appears to respond in a similar manner as A-substance to changes in the reagent. Comparisons of the colors obtained with A-substance and N-acetylglucosamine are described in the following sections.

*Conditions for the Color Development.* The concentration of HCl in the *p*-dimethylaminobenzaldehyde reagent is 0.24  $N$ ; this differs from the concentration, 0.6  $N$  HCl, recommended by Morgan and Elson. The latter concentration was unsuitable; the maximum color intensities for A-substance preparations with the reagent occurred in 10 minutes and the color faded rapidly thereafter at the temperature of development, 25°C. The lower concentration of HCl gave a maximum color intensity in 50–60 minutes for both A-substance and N-acetylglucosamine and the colors were stable for at least 30 minutes longer. The marked effect of temperature on development as well as HCl concentration on the rate of color development was noted by Morgan and Elson, who used temperatures of 13–16°C. For this reason the temperature during development was controlled by immersing solutions in a water bath thermostat at  $25.0 \pm 0.1^\circ C$ . Since HCl concentration influences the rate of color development, reliable results will be obtained only if the test solutions of preparations do not contain large amounts of buffering agents.

*Comparison of Colors Obtained with A-Substance and N-Acetylglucosamine.* Morgan and King (3) have suggested that the color reaction with Ehrlich's reagent is due to an N-acetylglucosamine rest in A-substance. A-substance from hog gastric mucin was found to give colors equivalent to 12–13% N-acetylglucosamine; this estimate

is approximately confirmed. The equivalent *per cent* N-acetylglucosamine of a number of purified A-substance fractions has been found to vary from 10-13%; these values were calculated for colors obtained with 1000  $\gamma$  of material and for a period of heating with alkali of 15 minutes for A-substance and 5 minutes for N-acetylglucosamine. The fractions were isolated from hog gastric mucin by fractionation with alcohol, sodium sulfate or phenol and alcohol, from pepsin by autolysis or alcohol fractionation, or by treatment of A-substance with papain-HCN or formamide. Morgan and King used results for shorter digestion times of A-substance (6 minutes) for their calculations; their test conditions also differ as has been discussed above. It is of interest that the equivalent *per cent* N-acetylglucosamine accounts for less than half of the 28-30% hexosamine found in A-substance (3, 8, 11).

It should be noted that calculations of equivalent N-acetylglucosamine content are subject to variation depending on the amount of A-substance used for analysis and on the time of digestion of A-substance and N-acetylglucosamine with sodium carbonate solution. A maximum color intensity occurs for most A-substance preparations after digestion at 100°C. with alkali for 15 minutes; a considerably sharper maximum is produced for N-acetylglucosamine after 5 minutes digestion with alkali.

#### *Serological Test (Inhibition of Isoagglutination)*

*Anti A-( $\alpha$ )-Agglutinin.* Pooled serum collected from persons of blood Group B was used. This serum was prepared by the Hyland Laboratories<sup>4</sup> and was selected for reasonable potency before its pooling. All of the serum used by us bore the same lot number. It was dyed with a preservative and stored at 5°C. in the original containers. The undiluted serum just produced incipient agglutination at a dilution of 1:128 when tested against pooled A cells by the method described below. The potency of the serum remained constant during the course of the experiments. The serum was diluted with 24 volumes of 0.9% NaCl solution just before use in the inhibition of isoagglutination studies. Thus, there were present 20 microliters of serum in each tube of a series, while 4 microliters of serum were sufficient to produce just microscopically visible agglutination of the volume of cells used in the inhibition system. This four-fold excess of serum over that required to give incipient agglutination was insufficient, however, to produce complete agglutination of the volume of pooled Group A cells (not selected with reference to subtype) used.

*Anti B-( $\beta$ )-Agglutinin.* Pooled serum from persons of blood group A was used. This serum, prepared by Hyland Laboratories, was similar to that containing anti A-( $\alpha$ )-agglutinin. The undiluted serum just produced incipient agglutination at a dilution of 1:256 when tested against pooled B cells by the method described below. As in the case of the anti A-( $\alpha$ )-agglutinin 20 microliters of group A serum were present in each tube of a series, with 2 microliters being sufficient to produce just microscopically perceptible agglutination of the group B cells present. Thus, there was employed a nine-fold excess of serum over that required for incipient agglutination. This amount was almost sufficient to produce complete agglutination of the volume of group B cells used.

*Group A Erythrocytes.* The cells used in each experiment were pooled from ten individuals, each of Group A. Since the source of the cells was a Red Cross blood bank

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<sup>4</sup> Address: 4534 W. Sunset Ave., Los Angeles, California.



center, each batch was obtained from different groups of donors. The unwashed cells were preserved at 5°C. and used until a suspension of them showed microscopically perceptible autoagglutination. They were then discarded. In no case were cells used for longer than 5 days following their collection from the donors. Just before use the cells were washed 4-5 times by suspending them in 4 times their volume of 0.9% NaCl solution, stirring and centrifuging. For use in the inhibition studies a 1% suspension of the freshly washed cells was prepared by diluting an aliquot of the packed cells with 0.9% NaCl solution.

*Group B Erythrocytes.* The pooled cells from ten individuals of blood group B were treated as described for group A cells.

*Experimental Procedure for Determinations of A and of B Activity.* The following description applies as well to the procedure for the determination of the inhibition of isoagglutination of group B cells as it does to that for group A cells, with suitable changes in the kind of sera and cells used. It is specifically discussed with reference to A activity only. Solutions of the substances to be tested were prepared in 0.9% NaCl at a concentration of approximately 1 mg./ml. Serial dilutions by two of the test substances with normal saline were made in small culture tubes. In making these dilutions 1 ml. serological pipets were used to mix and then withdraw 0.5 ml. aliquots from each tube for transfer to the next. A fresh pipet was used for each tube in a series. The final volume of test substance solution in each tube was 0.5 ml. Into each tube of the series and into suitable controls 0.5 ml. of 1:25 Group III (B) serum was pipetted. The contents of the tubes were mixed by sharp stroking of the tubes and were then allowed to stand in a water bath at 20-25°C. for one hour. One-half ml. of a 1% suspension of Group II (A) cells in normal saline was then pipetted into each tube of the series. The contents of each tube was again mixed and then allowed to stand for 2 hours at 20-25°C. At the end of this time the tubes were centrifuged for 1 minute in a clinical centrifuge. The examination of the mixture for agglutination was made as follows. The centrifuged cells in each tube in a series were resuspended by stroking the tube, at the same time observing whether there was massive agglutination. In this way it was possible to eliminate certain tubes from further consideration. For the final selection of the first tube in a series which had agglutination the contents were examined under the low-power objective of a microscope. The stage of the microscope was tilted so that when a drop of a cell suspension was streaked across the slide, the cells moved slowly across the field. By observing whether cells which seemed to be aggregated as they entered the field remained so as they moved across it, it was possible to distinguish true agglutination from adventitious clumping. The endpoint of inhibition was taken as the mean of the smallest amount of A substance which entirely prevented clumping of cells (recorded as 0 agglutination) and of that amount which permitted several 2-cell aggregates to be observed in two to three streakings of the suspension across the field (recorded as  $\pm$  agglutination). In some cases the first tube of a series to show agglutination exhibited it sufficiently strongly that 1-3 2-cell clumps were continually visible in the moving field (recorded as + 1 agglutination). In these latter cases, where there was no tube having  $\pm$  agglutination, the endpoint was taken as the mean of the amounts of test substance present in the last tube which showed 0 agglutination and in the first tube with + 1 agglutination.

The titer of a preparation, i.e., the microliters of serum neutralized  $\gamma$  of substance, was calculated by dividing 20 (the number of microliters of serum in the test system)

by the amount of material taken as the endpoint; the amount of serum which reacts with the red cells in the test system is neglected. The experiment described immediately below indicates that the titer of a preparation is essentially independent of the amount of serum used in the test system.

An experiment was performed to investigate the dependence of the inhibition titer of a test substance on the agglutinin concentration. Six sets of tubes were prepared, each set being of serial dilutions by two of an A-substance preparation in normal saline (final volume in each tube; 0.5 ml.). The test substance used in this experiment was material precipitated from hog gastric mucin by 30%  $\text{Na}_2\text{SO}_4$  and soluble upon electro dialysis of the precipitate.

To each tube within a series, 0.5 ml. of serum, diluted in some cases with normal saline, was added. A different dilution of serum was used for each series. After mixing their contents the tubes were allowed to stand 1 hour in a water bath at 25°C. To each tube 0.5 ml. of a 1% suspension of group A cells in normal saline was added. The tubes were allowed to stand for 2 hours at 37°C. They were then examined for agglutination by the method described above. The results are presented in Table I.

TABLE I  
*Dependence of Endpoint Upon Amount of Serum Used in Test*

Amount of Serum Present in Each Tube ( $\mu$ l)	Amount of Test Substance In Tube Showing Indicated Degree of Agglutination ( $\gamma$ )	
	0	$\pm$
500	36.1	18.1
250	18.1	9.0
125	9.0	4.5
62	2.2	1.1
31	2.2	1.1
20	1.1	0.56

Any endpoint obtained by the inhibition test may be in error by a factor of two. This limit of error is fixed partly by the use of serial dilutions by two and partly by the unknown extent to which small differences in experimental conditions affect the phenomenon of agglutination. With these considerations borne in mind the data in Table I can be seen to illustrate the generally linear dependence of inhibition endpoint on agglutinin concentration.

## RESULTS

The correlation of the serological activity and the color intensity obtained with Ehrlich's reagent for A-substance fractions prepared by mild procedures from hog gastric mucin<sup>5</sup> is shown in Fig. 1. The preparations were obtained by fractionation with sodium sulfate or

<sup>5</sup> Wilson Laboratories gastric mucin granules, Item No. 443.

phenol-alcohol according to the methods of Morgan and King (3), by alcohol fractionation, by aqueous extraction and by electrodialysis of some of the fractions derived from these procedures. The procedures do not subject A-substance to conditions which are known to degrade it, such as highly acidic or basic solutions at elevated temperatures (3).

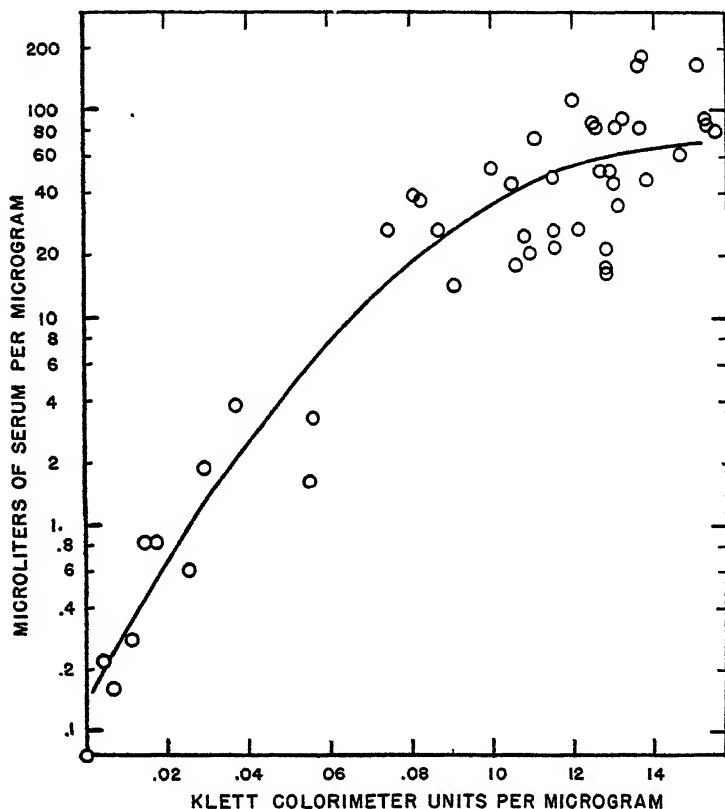


FIG. 1

Relation between Color Intensity with Ehrlich's Reagent and the Isoagglutination Inhibition Titer for A-Substance Preparations from Hog Gastric Mucin

This restriction permits a more accurate estimate of the degree of correlation between the color test and the serological test since degraded materials, which may differ substantially in behavior from undegraded materials in these tests, are omitted from consideration.

Data for 47 separate fractions are plotted in Fig. 1. The serological activity is reported as microliters of serum neutralized/ $\gamma$  of test material. The value of the titer for a preparation was calculated from the mean of the amounts of material found by the serial dilution technique to produce minimal agglutination and complete inhibition of agglutination. Usually only single determinations of titers were made; the precision of the determination is estimated to be  $\pm 50$ –100% over the whole range of titers.

The ability of the substance to give a color with Ehrlich's reagent is given on the abscissa of Fig. 1 as Klett Colorimeter units/ $\gamma$  of test material. This value is not entirely independent of the amount of material utilized in the color test, usually about 1000  $\gamma$  with an over-all range of about 500–1500  $\gamma$ . However, the values of the slopes (Klett Colorimeter units/ $\gamma$ ) at the limits of this range were found to differ by less than 8% from the slope for 1000  $\gamma$  of material for a number of A-substance preparations. Since these deviations approach the precision of the color test and are small compared to errors in the serological test, it would be expected that the existence of a correlation between the two tests would not be obscured by neglecting these deviations. No corrections for these deviations have been applied to the data in this study. Such corrections would be a desirable refinement as the precision of the methods improve and when the nature of the color reaction is clearly established. The majority of the slopes reported are the mean of two determinations with an average deviation from the mean of 3% for highly active preparations and as much as 10–30% for weakly active preparations (less than 0.5 microliters/ $\gamma$ ).

The curve shown in Fig. 1 is the least squares fit of a second-degree parabola to the data; the index of correlation for the curve is 0.95. The data show a normal distribution about the least squares curve; a semi-logarithmic plot of the data is convenient because of the wide range of titers of preparations. Since the precision of the color test is about ten times greater than the serological test (except at very low activities), the least squares solution has not considered the errors which may be involved in the colorimetric estimation. Because of the difficulty of estimating very faint colors at low activities, the estimated titers from the least squares curve below 0.17 microliters/ $\gamma$  are not significant.

The curve in Fig. 1 shows that a significant relation exists between the color with Ehrlich's reagent and blood group A activity. This

TABLE II  
*Comparison of Observed and Estimated Titers for Fractions  
 Isolated from Hog Gastric Mucin*

Compound No.	Procedure	Titer ( $\mu$ l serum/ $\gamma$ )		Probability of observed deviation
		Observed	Estimated	
R4-F4	Alcohol fractionation (Land- steiner and Harte)	0.085	0.17	0.27
R5-F1		.29	.20	.56
R4-F1		.30	.27	.87
R5-F2		20	19	.94
R10-F2		8.2	19	.18
R8-F2B		44	27	.43
R4-F2		29	28	.96
R5-F3		111	44	.14
R8-F1		54	54	1.00
R4-F3		63	56	.85
R11-F2	Procedure of Meyer, Smyth, and Palmer	154	59	.13
R8-F2A		42	61	.56
C-51		6.1	2.2	.11
C-49		5.7	8.0	.59
C-54		22	27	.75
C-8		27	35	.68
C-50		21	39	.33
C-4		53	44	.77
C-48		33	65	.28
C-75	Treatment with ammoniacal copper solution	84	45	.32
C-76		84	54	.49
C-77		44	55	.72
R14-F2A	Treatment with alkaline pyri- dine	48	38	.71
R14-F1A		53	53	1.00

\* Notes to Table II.—*R4-F4*: Soluble after addition of 2 vol. EtOH to filtrate of aqueous suspension of mucin. *R5-F1*: Insoluble in aqueous suspension of mucin, pH 4.3, after heating at 90°C. *R4-F1*: Same as R5-F1, duplicate experiment. *R5-F2*: Insoluble on addition of  $\frac{3}{4}$  vol. EtOH to filtrate of aqueous suspension of mucin. *R10-F2*: Obtained by dialysis of R5-F2 for two weeks at 5°C. *R8-F2B*: Residue on electro dialysis of R5-F3 for 5 days. *R4-F2*: Same as R5-F2, duplicate experiment. *R5-F3*: Precipitated between  $\frac{3}{4}$  and 2 vol. EtOH from filtrate of aqueous suspension of mucin. *R8-F1*: Clear supernatant on electro dialysis of R5-F3 for 5 days. *R4-F3*: Same as R5-F3, duplicate experiment. *R11-F2*: Obtained by dialysis of R5-F3 for two weeks at 5°C. *R8-F2A*: aqueous washing of R8-F2B. *C-51*: Precipitated at pH 7 by zinc acetate. Electro dialyzed at pH 5.3, insoluble after electro dialysis. *C-49*: After

TABLE II (Continued)

Compound No.	Procedure	Titer ( $\mu$ l serum/ $\gamma$ )		Probability of observed deviation
		Observed	Estimated	
R7-S1B	Treatment with HCONH <sub>2</sub> at 150°C. (Landsteiner and Harte)	.51	1.6	.075
R7-F3B		4.3	6.4	.53
R7-S2		11	26	.19
R7-F2A		18	38	.24
R7-F2C		9.7	47	.01
R7-S3		5.4	48	.0006
R7-F2B		11	54	.01
R7-F1		29	64	.21
R7-F4A		7.1	64	.0006
R7-F5A		12	65	.008
R6-F4A	Treatment with papain-HCN (Landsteiner and Harte)	6.9	56	.001
R6-F1A		37	59	.46
R6-F2		48	60	.72
R6-F1B		24	63	.13
R6-F5A		25	65	.13
1A	A-substance preparation by Kabat and Bezer	87	64	.63
M-330	A-substance preparation by Lilly	83	60	.61
960-GM-2	A-substance preparations by Sharp and Dohme	77	57	.64
960-GM-1B		91	55	.43
960-GM-1C		77	59	.67

precipitation by 18 vol. glacial acetic acid, insoluble in water, insoluble after electro-dialysis. C-54: As for C-51, except soluble after electro-dialysis. C-8: Obtained after initial precipitation with 3 vol. EtOH, and after precipitation with 18 vol. glacial acetic acid, not dialyzed. C-50: As for C-49, except soluble after electro-dialysis. C-4: Obtained after the initial precipitation with 3 vol. EtOH, but before acetic acid precipitation, not dialyzed. C-48: Obtained after precipitation by 18 vol. glacial acetic acid. Material soluble in water and precipitated at 30-35°C. by 30% Na<sub>2</sub>SO<sub>4</sub>, electro-dialyzed. C-75: Extracted by phenol from mucin and treated for 1 hr. at room temperature with an ammoniacal copper solution (reagent contained 4.95 g. Cu/L and was 5.2 M in NH<sub>4</sub>OH). Adjusted to pH 5 with acetic acid. Dialyzed at room temperature. C-76: As for C-75, except treated 6 hr. with the reagent. C-77: As for C-75, except treated for 3 hr. with the reagent. R14-F2A: A-substance, isolated by Na<sub>2</sub>SO<sub>4</sub> fractionation, treated with alkaline pyridine (0.002 M NaOH in 90% pyridine) for 2 days and then the material soluble in alkaline pyridine precipitated with acetone, dialyzed. R14-F1A: As for R14-F2A except material was insoluble in alkaline pyridine, dialyzed. R7-S1B: Soluble on addition of 2 vol. EtOH to formamide solution of

curve can be considered only as an approximation; more data, particularly at low and moderate activities, would be required for a more accurate relationship. Fig. 1 has been used for the estimation of the blood group A activity of, (a) fractions obtained from hog gastric mucin by somewhat more drastic conditions (Table II); (b) A-substances prepared by other investigators from hog stomach or hog gastric mucin (Table II) and (c) fractions obtained from sources other than hog stomach (Table III). Brief descriptions of the treatment and method of isolation of fractions are given in notes to the tables.

Table II shows a comparison of the observed and estimated titers for fractions isolated from hog gastric mucin<sup>6</sup> by alcohol fractionation of a previously heated mucin suspension (8), by treatment with ammoniacal copper solution, alkaline pyridine, formamide (8), papain-HCN (8) or by the procedure of Meyer, Smyth and Palmer (11), which involves a preliminary treatment of mucin with sodium carbonate at 70°C. Comparison of observed and estimated titers are also shown for A-substance isolated by Kabat and Bezer (1) from hog

R5-F3. Dialyzed. Soluble in water on dialysis. R7-F3B: Soluble on addition of 4 vol. EtOH to solution of R7-F2B. R7-S2: Supernatant from first reprecipitation of R7-F1 from 66% EtOH, dialyzed. R7-F2A: Insoluble in 90% acetic acid solution of R7-F1. R7-F2C: Supernatant from the precipitation of R7-F2B from 90% acetic acid with acetone, dialyzed. R7-S3: Supernatant from second reprecipitation of R7-F1 from 66% EtOH, dialyzed. R7-F2B: Precipitated on addition of  $\frac{1}{2}$  vol. of acetone to supernatant of 90% acetic acid solution of R7-F1, dialyzed. R7-F1: Insoluble on addition of 2 vol. EtOH to formamide solution of R5-F3. Reprecipitated twice from 66% EtOH. R7-F4A: Insoluble on addition of 4 vol. EtOH to solution of R7-F2B. Reprecipitated from HCl solution with 4 vol. EtOH, dialyzed. R7-F5A: Precipitated from 90% acetone solution of R7-F4A. R6-F4A: Insoluble on addition of 4 vol. EtOH to solution of R6-F1A. Insoluble in 90% acetic acid solution. R6-F1A: Insoluble on addition of 1.5 vol. EtOH to filtered and dialyzed papain-HCN digest (7 days at 37°C.) of R4-F3. R6-F2: Supernatant from the precipitation of R6-F1A and R6-F1B with EtOH. R6-F1B: Same as R6-F1A except this fraction did not centrifuge readily from alcohol solution and was obtained by Seitz filtration. R6-F5A: Insoluble on addition of 4 vol. EtOH to solution of R6-F1A. Soluble in 90% acetic acid solution, precipitated by acetone, and dialyzed. 1A: Prepared by alcohol fractionation, see reference (1). M-330: Prepared from hog stomach linings by peptic autolysis and alcohol fractionation. 960-GM-2: Prepared from hog gastric mucin by phenol fractionation. 960-GM-1B: Prepared by autolysis from hog gastric mucin. 960-GM-1C: Prepared by tryptic digestion and alcohol fractionation from hog gastric mucin.

<sup>6</sup> Except where otherwise indicated, the hog gastric mucin was obtained from Wilson Laboratories (Item No. 443).

TABLE III  
Comparison of Observed and Estimated Titers for Fractions  
Isolated from Sources other than Hog Gastric Mucin\*

Compound No.	Source	Procedure	Titer ( $\mu$ l serum/ $\gamma$ )		Probability of observed deviation
			Observed	Estimated	
C-105	Horse stomach mucosa; (B activity, $<.04 \mu$ l/ $\gamma$ )		0.21	0.33	.48
M-336C	Horse stomach prepared by Lilly; (B activity, $4.8 \mu$ l/ $\gamma$ )		3.2	9.8	.08
960-P-HS-4	Horse stomach linings prepared by Sharp & Dohme; (B activity, $5.6 \mu$ l/ $\gamma$ )		.44	34	.0000
DM	Duodenal mucosa (Wilson)	Untreated source material	.87	.22	.03
C-32	Pepsin (Parke, Davis)		.40	.19	.24
C-33	Pepsin (Wilson)		1.9	.28	.003
C-5	Pepsin	Autolysis procedure of Landsteiner and Harte	2.0	.66	.08
C-22			61	48	.69
C-44	Pepsin	Alcohol fractionation (Landsteiner and Chasc)	.049	0	—
C-26			27	52	.30

\* Notes to Table III.—*C-105*: From fresh horse stomach mucosa treated according to U.S. Patent 1,829,270 (S. J. Fogelson) and then according to the  $\text{Na}_2\text{SO}_4$  fractionation method of Morgan and King (3). *M-336C*: Prepared by peptic hydrolysis and alcohol fractionation. *960-P-HS-4*: Prepared by peptic-tryptic digestion and alcohol fractionation. *DM*: Wilson Laboratories duodenal mucosa preparation. *C-32*: Parke, Davis and Co. pepsin (1:3000). *C-33*: Wilson Laboratories pepsin (1:10,000), Item No. 414. *C-5*: From Wilson pepsin (see *C-33*), autolyzed, precipitated by 66% EtOH and then dissolved. Finally precipitated by 50% EtOH. *C-22*: From Wilson pepsin (see *C-33*), autolyzed, precipitated by 62% EtOH and then by 9 vol. glacial acetic acid and 5 vol. acetone; dialyzed and precipitated by 4 vol. EtOH plus sodium acetate. *C-44*: From Wilson pepsin (See *C-33*), precipitated by acetic acid from aqueous solution, washed. *C-26*: From Wilson pepsin (see *C-33*), obtained



TABLE III (Continued)

Compound No.	Source	Procedure	Titer ( $\mu$ l serum/ $\gamma$ )		Probability of observed deviation
			Observed	Estimated	
C-27	Blood group A stromata	Procedure of Kossjakow	2.4	6.8	.10
R1-F1	Blood group A stromata	Procedure of Hallauer	<.008	0	—
R1-F3			<.008	0	—
C-45			.18	0	—
R1-F5			<.14	.18	—
R1-F2			.35	.19	.34
R1-F4			.067	.20	.08
C-30			.067	.26	.03
C-28			.18	.28	.49
C-73			.77	.56	.61
C-34	Blood group A erythrocytes	Procedure of Hallauer	.011	0	—
C-2			<.09	0	—
C-46			.13	0	—
C-57			<.003	0	—
C-3			<.09	.18	—
C-56			.92	.21	.02
C-1			.083	.24	.09
C-113	Pseudo-mucinous ovarian cysts (blood group A)	Alcohol fractionation	.42	0	—
C-119			1.8	2.5	.60

by two 65% alcohol precipitations at pH 8.5, dialyzed. C-27: Treated according to Kossjakow's procedure as far as one alcohol precipitation. R1-F1: Extract with 95% EtOH. R1-F3: First 25% EtOH extract. C-45: Boiling absolute alcohol extract. R1-F5: Extract with 10% EtOH. R1-F2: Extract with 50% EtOH. R1-F4: Second 25% EtOH extract. C-30: First 25% EtOH extract. One acetone precipitation. C-28: Second 25% EtOH extract. One acetone precipitation. C-73: Aqueous extract. Precipitated once with acetone. C-34: Extract with 10% EtOH. One acetone precipitation. C-2: First 25% EtOH extract. One acetone precipitation. C-46: Boiling absolute alcohol extract. C-57: Material precipitated by 66% EtOH from aqueous extract of material extracted by 50% EtOH according to Hallauer's procedure. C-3: Second 25% EtOH extract. One acetone precipitation. C-56: Material precipitated by 50% EtOH from aqueous extract of material extracted by 50% EtOH according to Hallauer's procedure. C-1: Extract with 50% EtOH. One acetone precipitation. C-113: Material precipitated by 50% EtOH from the native fluid of a pseudomucinous

gastric mucin and for A-substances isolated from hog stomach and hog gastric mucin by the Sharpe and Dohme and Lilly laboratories.<sup>7</sup>

Column 5 in Table II gives the calculated probability that the deviations between observed and estimated titers are due to a normal distribution of error. Probabilities less than 0.05 indicate deviations from the estimated titers which are significantly larger than deviations from the least squares curve shown in Fig. 1. The agreement between observed and estimated titers is satisfactory except for some of the fractions isolated by formamide and papain procedures. However, these two procedures are known to cause extensive degradation of A-substance (3, 8) and to decrease the isoagglutination titer of preparations. In these cases it appears that the degradation has disturbed the structure of A-substance to such a degree that the serological specificity is lost while the functional groups responsible for the color test are relatively unaffected. The other procedures of isolation apparently effect no substantial degradation of A-substance detectable by means of a comparison of the observed and estimated titers.

Table III summarizes the results of observed and estimated blood group A titers for fractions isolated from pepsin (8, 9), blood-group A erythrocytes and stromata (12, 13), horse stomach, and pseudo-mucinous ovarian cyst fluids of individuals of blood group A.<sup>8</sup> Materials isolated from horse stomach mucosa are often known to possess blood group B activity; titers against B cells are shown parenthetically in Table III for these preparations. In several cases unpurified materials were analyzed by the serological and color tests; comparisons of these results would not be expected to be as satisfactory as in those cases where some means has been resorted to for the separation of inactive materials which may interfere with the tests.

Probabilities are not calculated for those cases in which the estimated titer is below the limit of the colorimetric method, 0.17 microliters/ $\gamma$ , or in those cases where the observed titer is only known to be less than

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ovarian cyst removed from an individual of blood group A. Material dialyzed. C-119: Material soluble in 50% EtOH but insoluble in 65% EtOH from same cyst fluid as C-113. Material reprecipitated once and dialyzed.

<sup>7</sup> We are indebted to Dr. E. Brand for a sample of Kabat's preparation, and to Dr. R. H. Barnes of Sharpe and Dohme, Inc., and Dr. J. A. Leighty of Lilly Research Laboratories for other samples of A-substance and AB mixture.

<sup>8</sup> We are indebted to Dr. R. W. Hammack of the Hospital of the Good Samaritan, Los Angeles, California, for providing us with samples of fluids removed from ovarian cysts.

a given value. These results are of interest since they indicate that the colorimetric method is usually in qualitative agreement with the serological test for fractions of no, or very low, activity.

The results for material isolated from horse stomach are pertinent to the question of the behavior of B-substance isolated from this source in the color test. Recently Morgan and Waddell (14) have observed that B and O substances isolated from pseudo-mucinous ovarian cyst fluids give color tests after treatment with alkali. The agreement between the observed and estimated blood group A titers for the horse stomach preparations shown in Table III is satisfactory either when the B activity of the substance is low (Compound C-105) or when the serological test shows appreciable A activity (Compound M-336C). When a compound shows appreciable B activity but little A activity (Compound 960-P-HS-4), the color test still predicts a very high A activity, the agreement in this case being very poor. This evidence would appear to suggest that B-substance or some material associated with it in horse stomach also gives the color test or that the A-substance factor has been almost completely degraded by the peptic-tryptic digestion used in the preparation of this compound.

The deviations between observed and estimated titers for the remainder of the results in Table III have calculated probabilities which are usually greater than the value of 0.05 of the standard of significance. Some of the probabilities fall somewhat below 0.05 for preparations of low activities; this might be expected, as errors in the colorimetric procedure, which were not considered in the least squares solution, become appreciable at low color intensities.

## DISCUSSION

The results which have been presented indicate that the functional groups responsible for the color reaction with Ehrlich's reagent are an inherent feature of the structure of A-substance. The groups appear to be common to the materials possessing A activity derived from human erythrocytes and pseudo-mucinous ovarian cyst fluids as well as from hog gastric mucin, pepsin and horse stomachs. It would not be expected that detailed differences in the A-substances isolated from these various sources could be revealed in this study due to the lack of precision in the correlation of the serological test and the color test.

Preliminary evidence from materials isolated from horse stomach would appear to indicate that B substance isolated from this source

also possesses similar functional groups; further investigation is required to establish these observations. It is evident that a knowledge of the specificity of the color reaction is important as a part of the general problem of interpreting serological specificity in terms of the chemical structure of the blood group substances.

While the colorimetric procedure has proved to be a useful supplement to serological tests in our work, it may be helpful to point out several difficulties in applying the procedure in its present form as a general method for the estimation of isoagglutination titers of A-substance preparations. First, the method requires a preliminary calibration with the aid of the relatively inaccurate serological test in case one is interested in interpreting the results in terms of serological titers. The empirical relation which is derived in this way will probably be difficult to obtain in a more direct manner until further investigation establishes the chemical structure of A-substance. Another feature restricting the usefulness of the colorimetric method is the fact that the estimated isoagglutination titers may be considerably in error if A-substance has been degraded significantly. This limitation imposes the necessity of determining by preliminary investigation whether the colorimetric method is suitable for the prediction of isoagglutination titers of fractions obtained by various procedures.

Finally, certain limitations in the color test restrict a routine application of the method. For example, solutions of preparations must be essentially neutral and contain no significant amount of buffer since the adjustment of the pH during the sodium carbonate digestion of A-substance as well as on addition of Ehrlich's reagent is critical. Occasionally preparations give turbidities in the final solutions and thus prevent accurate measurements of color intensity. Difficulties of this kind could probably be resolved by altering the experimental conditions of the color test.

#### SUMMARY

The intensity of the color produced by the reaction of A-substance with Ehrlich's *p*-dimethylaminobenzaldehyde reagent after preliminary treatment of A-substance preparations with alkali has been correlated with the isoagglutination inhibition activity of the original preparations. The use of this correlation to estimate the potency of A-substance preparations has been described. The color reaction with Ehrlich's reagent has been observed for blood group A specific preparations

isolated from hog gastric mucin, pepsin, blood group A erythrocytes and stromata, and human ovarian cyst fluids, and for blood group AB specific preparations from horse stomachs.

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# A Precision Method for the Quantitative Determination of Calcium in Blood Plasma \*

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## INTRODUCTION

Cerium salts as oxidizing agents are gradually replacing permanganate for use in analytical chemistry. However, their application to analytical procedures in biological chemistry has been more slowly forthcoming. For the estimation of calcium in blood plasma, the use of ceric sulfate as oxidant of the oxalate equivalent was first suggested by Rappaport and Rappaport (11) and later modified by Larson and Greenberg (6) and Kirk and collaborators (4, 7). In most instances an excess of ceric sulfate was added to a solution containing the oxalate equivalent of calcium and the excess ceric sulfate was titrated back with ferrous ammonium sulfate. The oxalate ion has been titrated directly with ammonium hexanitratocerate in perchloric acid solution using *o*-phenanthroline ferrous sulfate (ferroin) as indicator (3). The same procedure has been employed on a macro scale with nitro-*o*-phenanthroline ferrous sulfate (nitroferroin) instead of *o*-phenanthroline ferrous sulfate as the indicator (13).

In regard to the preparation of the serum for subsequent calcium analysis, a detailed study has been made by Sendroy (12). He concludes that accurate values may be obtained by direct precipitation but that preliminary ashing is theoretically the best. He points out, however, that direct precipitation might not be a feasible procedure if fatty or opaque sera have to be analyzed. Some evidence will be presented in this paper concerning this point.

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This report is concerned mainly with the determination of calcium in plasma, using 0.5 ml. samples for analysis. In brief, the procedure consists of the preliminary treatment of the plasma by ashing with a perchloric acid-nitric acid mixture, the precipitation of calcium as calcium oxalate, and the titration of the oxalate ion with ammonium hexanitratocerate in perchloric acid solution using nitro-*o*-phenanthroline ferrous sulfate as indicator.

### REAGENTS

1. 72% C. P. perchloric acid, sp. gr. 1.69.
2. 2 *N* perchloric acid. (167 ml. of 72%  $\text{HClO}_4$  diluted to 1000 ml. with redistilled water. Double distilled  $\text{HClO}_4$  is preferred but not essential.)
3. Concentrated nitric acid, sp. gr. 1.42, analytical reagent.
4. Ashing mixture: 1:1 mixture of 72% perchloric acid and concentrated nitric acid.
5. 0.04 *N* hydrochloric acid.
6. Triton N. E. (1:30 dilution of the original solution obtained from Rohm and Haas Co., Washington Square, Philadelphia.)
7. 2% ammonium hydroxide. Prepared daily. (It is suggested that the concentrated ammonium hydroxide be distilled and a 10% stock solution made from which the 2% solution is obtained.)
8. Saturated ammonium oxalate, analytical reagent. Kept at room temperature.
9. 0.001 *N* sodium oxalate, primary standard, analytical reagent. The salt is dissolved in 2 *N* perchloric acid. In the absence of perchloric acid the solution is not stable on storage in this concentration.
10. 0.00025 *N* nitro-*o*-phenanthroline ferrous sulfate indicator. (May be obtained from the G. Frederick Smith Chemical Co., Columbus, Ohio, in 0.025 *M* solution—to be diluted with redistilled water.)
11. Ammonium hexanitratocerate, standard or reference purity (G. Frederick Smith Chemical Co.). A 0.01 *N* solution of the cerium salt in 2 *N* perchloric acid is first prepared, as follows: Treat 5.4819 g. ammonium hexanitratocerate by adding slowly to the salt 167 ml. of 72% perchloric acid, stirring constantly. Then add slowly, with constant stirring, redistilled water to 1000 ml. The 72% perchloric acid is added first to promote the formation of perchloratoceric acid by replacement of the nitrate radical of nitratoceric acid. If the solution is not made up in this way an insoluble precipitate of a mixed nitratoperchloratoceric acid may deposit on standing. Slow addition of 50 ml. of water with intermediate stirring for one minute after each addition is requisite. This resulting 0.01 *N* perchloratoceric acid solution is diluted with 2 *N* perchloric acid to a 0.001 *N* concentration which is standardized with 0.001 *N* sodium oxalate and used in the final titration procedure. It should be stored in the dark and in the refrigerator to increase its stability with age.

### PROCEDURE

*I. Ashing.* 0.5 ml. plasma or serum is pipetted into a 25 ml. pyrex Erlenmeyer flask and 2 ml. of the ashing mixture added. The flask is placed on a hot plate ad-

justed to a temperature range of approximately 170–250°C. Ashing is begun at a temperature of 180°C. (If plasma of high lipid content is to be ashed, an initial temperature of about 100°C. is recommended to avoid spattering.) The temperature is raised slowly to about 240°C. and should never exceed 250°C. The ashing is completed when there are no remaining drops of liquid, no more white fumes escaping, and when only the snow-white dry ash remains in the flask. It is important that no traces of HClO<sub>4</sub> are left in the flask because the presence of the perchlorate ion interferes with quantitative precipitation of calcium oxalate in the order of magnitude with which the described procedure is concerned. The ashing process takes about 3–4 hours which may be shortened by brushing the top half of the reaction flask with a small colorless gas flame at the end of the digestion to minimize refluxing of the acid condensate on the inside walls of the 25 ml. Erlenmeyer flask and facilitate elimination of the excess acid.

*II. Precipitation of Calcium as Calcium Oxalate.* The ash is dissolved while still hot with 2 ml. of 0.04 *N* HCl. If the ash does not dissolve immediately, gentle heating over a micro flame will complete solution. The dissolved ash is transferred with redistilled water to a conical 15 ml. centrifuge tube. The total volume of water used for several washings should be approximately 5 ml.

To the dissolved ash in the centrifuge tube, 2 ml. of saturated ammonium oxalate are added. The mixture is left for 16 hours at room temperature for complete precipitation (2). No pH adjustment is necessary if the amounts and concentrations indicated above are employed. We have tested the pH of these solutions in numerous instances and have always found it to be between 4.0 and 6.0. This is within the optimal range for the precipitation of calcium oxalate as indicated by Sendroy (12).

*III. Treatment of the Precipitated Calcium Oxalate.* The manipulation of the precipitate follows the principles indicated by Sendroy (12), with slight modifications.

To the solution containing the calcium oxalate precipitate, 0.2 ml. Triton is added before centrifuging at 2600 r.p.m. for 5 minutes. The supernatant liquid is slowly siphoned off with a hooked capillary, allowing about 0.2 ml. of the liquid to remain in the tube. The precipitate is washed with 3 ml. of 2% NH<sub>4</sub>OH, allowing 1 ml. of the NH<sub>4</sub>OH to wash down the sides of the tube slowly, while the remaining 2 ml. may be added more rapidly—again down the sides of the tube. Centrifuging is repeated at 2600 r.p.m. for 5 minutes. The supernatant liquid is drawn off as before and washed for the second time in the same way.

After the third centrifugation and withdrawal of the fluid, the precipitate is immediately dissolved with 4 ml. of 2 *N* HClO<sub>4</sub>, while stirring. The solution is transferred to a 25 ml. beaker and the centrifuge tube washed twice with a total of 6 ml. of 2 *N* HClO<sub>4</sub>, thus giving a final volume of 10 ml. in the beaker.

*IV. Titration.* Immediately before titration, 0.5 ml. of 0.00025 *N* nitro-*o*-phenanthroline ferrous sulfate indicator is added, producing a distinct pink color. A 0.001 *N* perchloratoceric acid solution is used for the oxidation of the oxalate ion. The end point is marked by a sharp color change from pink to colorless.

The titration is carried out with a 10 ml. microburette (Koch) which is graduated in 0.05 ml. and has a reservoir and standard capillary tip.

One ml. of 0.001 *N* perchloratoceric acid solution is equivalent to 20  $\gamma$  of calcium.



## STANDARDIZATION OF THE PERCHLORATOCERIC ACID SOLUTION

The standardization of the 0.001 *N* perchloratoceric acid solution in 2 *N* perchloric acid is carried out in the same manner as the titration of the oxalate from calcium oxalate. A 0.001 *N* solution of Bureau of Standards sodium oxalate in 2 *N* perchloric acid is utilized in this standardization. For 10 cc. volume, 0.5 ml. of 0.00025 *N* nitroferroin indicator is employed. The blank correction for the indicator thus amounts to approximately 0.1 ml. of oxidant. The indicator correction can be repeatedly and easily duplicated precisely. The amount of indicator correction, therefore, introduces no error.

The stability of perchloratoceric acid solutions in the 0.001 *N* range was tested under various conditions and the data obtained are represented in Table I. The

TABLE I

*Stability of Perchloratoceric Acid Solutions in the 0.001 Normality Range*

	Day	Solution kept in light	Solution kept in dark	Solution kept in dark at about 0°C.
Solution I.	1st	<i>N</i> 0.00130	<i>N</i> 0.00130	<i>N</i> 0.00130
	4th	0.00108	0.00130	0.00130
	5th	0.00105	0.00126	0.00129
	6th	—	0.00125	0.00128
Solution II.	1st	0.00128	0.00128	0.00128
	4th	0.00120	0.00128	0.00128
	5th	0.00114	0.00127	0.00128
Solution III.	1st	0.00113	0.00113	0.00113
	3rd	0.00103	0.00113	0.00113
	4th	0.00098	0.00112	0.00113
	5th	0.00090	0.00112	0.00112

solutions were found to be stable over a period of approximately 4 days when kept in the dark at approximately 0°C. This stability is inferior to that of cerium salt solutions of higher concentrations which are known to be stable over a period of about 14 days (14). However, the stability of a 0.001 *N* perchloratoceric acid solution is superior to that of an equivalent permanganate solution.

## MODIFICATION OF PROCEDURE USING 3 ML. OF PLASMA

When larger samples are available for calcium determination as in the case of clinical routine analyses the following modification of the procedure outlined above has given very satisfactory results. Three ml. of plasma or serum are pipetted into a 25 ml. vitreosil crucible and dried at about 100°C. The sample is then ashed with a Bunsen burner until nearly all the material is white. Ashing is completed by adding

2 ml. of the perchloric acid-nitric acid mixture and placing the sample on a hot plate at about 240°C. until a dry white ash is obtained. The total time needed for drying and ashing is about 2 hours.

The ash is dissolved in 5 ml. of 0.04 *N* HCl and the solution transferred with a total of 15 ml. of water to a 40 ml. centrifuge tube. Four ml. of saturated  $(\text{NH}_4)_2\text{C}_2\text{O}_4$  are added and the mixture left for 16 hours at room temperature. No pH adjustment is necessary. The treatment of the precipitated calcium oxalate is identical with that described above except that 4 ml. of 2%  $\text{NH}_4\text{OH}$  are used for each washing. For the final titration procedure a 0.01 *N* perchloratoceric acid solution is employed. However, the amount and concentration of the indicator nitroferroin is the same as that used with 0.001 *N* perchloratoceric acid.

### EXPERIMENTAL RESULTS

To determine the sensitivity and accuracy of microtitrations of the oxalate ion using 0.001 *N* perchloratoceric acid with nitroferroin as indicator, oxalate equivalents of various concentrations of calcium were titrated. The results of these titrations corresponding to a range of 10–120 micrograms of calcium are given in Table II. The titration procedure showed a satisfactory accuracy in the desired range as the maximum standard error was  $\pm 0.5\%$ .

Investigations followed concerning possible interference of  $\text{HClO}_4$ – $\text{HNO}_3$  ashing with the accuracy of the subsequent titration. Samples of  $\text{CaCl}_2$  solutions of known concentration were evaporated to dryness with the  $\text{HClO}_4$ – $\text{HNO}_3$  mixture, calcium was precipitated as  $\text{CaC}_2\text{O}_4$  as described and, finally, the oxalic acid equivalent was titrated. The values obtained are shown in Table III. The standard error did not exceed  $\pm 0.53\%$  and the results were considered satisfactory.

Table IV contains the results of calcium analyses of ashed blood plasma after the addition of varying quantities of calcium. Plasma from chickens and humans was used in these experiments. The data of Table IV reveal that recovery was satisfactory.

That the direct precipitation of calcium as calcium oxalate in sera of high lipid content might not reveal accurate values has been emphasized by Tingey (15). Sendroy (12) analyzed a number of "fatty, more or less opaque" sera and obtained closely similar calcium values employing the method of direct precipitation as well as that of precipitation after ashing. He points out, however, that the number of analyses reported is not sufficient for decisive conclusions and that the problem of the interference of lipids with the direct precipitation of calcium warrants further research.

TABLE II  
Accuracy and Range of Oxalate Titrations

Calcium equivalent $\gamma$	Calcium found $\gamma$	Standard error	
		$\pm \gamma'$	$\pm \gamma''$
120	119.7	0.17	0.14
	119.7		
	120.4		
	120.4		
	120.4		
	Mean: 120.1		
60	60.0	0.05	0.08
	59.7		
	60.0		
	60.0		
	59.9		
	59.9		
30	Mean: 59.9		
	30.1	0.04	0.13
	30.0		
	29.8		
	30.0		
	29.9		
20	30.0		
	29.9		
	Mean: 30.0		
	19.9	0.04	0.20
	20.1		
	19.9		
10	19.9		
	19.8		
	19.9		
	Mean: 19.9		
	9.9	0.05	0.50
	10.1		
	10.1		
	9.9		
	9.9		
	Mean: 10.0		

TABLE III

*Determination of Calcium in Calcium Salt Solutions After Evaporation  
with  $\text{HClO}_4\text{-HNO}_3$  Mixture*

Amount of calcium present micrograms	Amount of calcium found micrograms	Standard error	
		micrograms <sup>1</sup>	$\pm\sigma\%$
242.4	242.5	1.00	0.41
	241.1		
	241.2		
	245.4		
	Mean: 242.6		
181.8	180.5	0.45	0.25
	181.9		
	181.0		
	183.1		
	182.1		
	Mean: 181.7		
121.2	119.9	0.64	0.53
	120.4		
	122.0		
	123.0		
	121.5		
	Mean: 121.4		
60.6	60.6	0.23	0.38
	60.3		
	61.0		
	59.9		
	Mean: 60.4		
20.3	20.2	0.07	0.35
	20.3		
	20.1		
	20.0		
	20.4		
	Mean: 20.2		

<sup>1</sup> Calculated as in Table II.

TABLE IV

*The Recovery of Calcium Added to Chicken and Human Plasma*

Calcium content of 0.5 ml. plasma micrograms	Calcium added micrograms	Total calcium present micrograms	Total calcium recovered micrograms	Recovery per cent
49.0	20.1	69.1	69.0	99.9
49.0	20.1	69.1	69.3	100.3
55.8	58.2	114.0	114.4	100.3
55.8	58.2	114.0	114.4	100.3
41.9	58.2	100.1	100.2	100.1
41.9	58.2	100.1	100.2	100.1
39.8	58.2	98.0	97.6	99.6
39.8	58.2	98.0	97.6	99.6
46.4	58.2	104.6	104.7	100.1
46.4	58.2	104.6	104.5	99.9
45.0*	58.2	103.2	102.4	99.2
45.0*	58.2	103.2	102.7	99.5
45.0	58.2	103.2	102.4	99.2
45.0 <sup>1</sup>	58.2	103.2	103.4	100.2
47.4*	60.6	108.0	107.2	99.3
47.4*	60.6	108.0	107.2	99.3
47.4*	60.6	108.0	107.4	99.4
47.4 <sup>1</sup>	60.6	108.0	107.2	99.3
43.2*	60.6	103.8	102.7	99.0
43.2*	60.6	103.8	103.0	99.2
43.2*	60.6	103.8	103.7	99.9
43.2 <sup>1</sup>	60.6	103.8	103.4	99.6

\* Human plasma.

It is a known fact that biological material containing fatty substances is rather difficult to ash. Since the  $\text{HClO}_4\text{-HNO}_3$  mixture used for the procedure reported in this paper proved highly efficient for fatty sera, it was thought that this technique might be advantageously applied to an analytical study concerning the determination of calcium in the presence of increased amounts of lipids. That fowl respond to the injection of appropriate amounts of estradiol benzoate with a marked lipemia and a concomitant rise of the blood serum calcium level has been shown by various workers (5, 10, 16). We have used this procedure to secure plasma of a high fat content; lipemia and calcemia were produced in 9 pigeons by the injection of 0.58 mg. of  $\alpha$ -estradiol benzoate over a period of 7 days. Three pigeons, which were

not injected, served as controls. As an indication of the lipid content of the plasma, cholesterol was determined by Bloor's technique (1) with slight modifications. Calcium was determined in plasma of high lipid content as well as in normal plasma by direct precipitation and by precipitation after ashing.

The cholesterol values for the plasma of the experimental animals, as well as the results of the calcium determinations obtained by the method of direct precipitation and those of precipitation after ashing, respectively, are as represented in Table V. Whereas the calcium

TABLE V

*Determination of Blood Calcium by Direct Precipitation and After Ashing, and Blood Cholesterol Values of Normal and Lipemic Pigeons*

Number of Pigeon	Controls			Injected with 0.58 mg. $\alpha$ -estradiol benzoate								
	54	5b	62	59	69	79	89	99	65	75	85	95
Calcium by Direct Precipitation mg. %	8.62	9.85	8.21	44.61	36.90	47.76	37.65	43.92	37.93	43.14	41.06	38.38
Calcium after Ashing mg. %	8.68	9.87	8.20	37.28	38.90	38.62	28.06	30.99	29.64	32.62	30.76	33.54
Cholesterol mg. %	398	395	365	775	614	505	735	685	595	900	730	688

analyses for the plasma of the control birds showed excellent agreement for the two methods employed, widely varying calcium values were obtained for the high lipid-containing plasma of the injected pigeons depending on the type of procedure utilized. Direct precipitation revealed higher calcium values than the determination of calcium after ashing in all plasma with a high lipid content. The values obtained in the plasma of normal birds are in good agreement with those reported in the literature (8, 9).

#### REMARKS

The wet ashing procedure involves the use of a mixture in equal proportions of conc.  $\text{HNO}_3$  (68%) and 72%  $\text{HClO}_4$ . A large excess is employed over the amount required to oxidize the organic matter in the sample taken for analysis. The reaction mixture is allowed to digest on a hot plate capable of producing a maximum temperature of about 250°C. As the reaction mixture rises in temperature the first oxidation effect is due to the  $\text{HNO}_3$ . All of the most easily oxidized organic matter is thus destroyed with accompanying evolution of considerable quantities of NO as indicated

by the color of the escaping fumes. The temperature of the mixture, because of the presence of an excess of  $\text{HNO}_3$ , is under  $160^\circ\text{C}$ . This fact is made evident because of the absence of white fumes of  $\text{HClO}_4$ , which begin to appear at  $160^\circ\text{C}$ . At this point  $\text{HClO}_4$  begins to exert its oxidizing effect. The last small portion of organic matter is then oxidized through the influence of hot  $\text{HClO}_4$ . As the reaction proceeds, the temperature gradually rises to  $200^\circ\text{C}$ . at which point a constant boiling mixture of perchloric acid and water results (72.5% perchloric acid). During this process of gradual concentration even the most difficultly oxidizable organic material is destroyed. By virtue of this stepwise oxidation of organic matter, no violent reactions are possible. A convenient oxidation vessel is a 25 ml. Erlenmeyer flask. The reason this flask is preferred is the fact that the volatilization of the distilling acid is retarded by a generous refluxing of the material on the cooler neck of the flask. This condition favors maximum utilization of the acid mixture in the oxidation of organic matter and prevents too rapid elevation of the temperature during the process of digestion. At the completion of the oxidation of the organic matter, the evaporation is continued to complete dryness and the total absence of volatile white fumes.

The use of  $\text{HClO}_4$  as acidifying reagent for the titration of the oxalate ion is not new. It has been used on a macro scale by Smith and Getz (13). On a micro scale it has been employed by Ellis (3), and Kirk and Tompkins (4). The latter two investigators failed to take advantage of the very important application of a high potential indicator such as nitroferroin as a preferred reagent compared to ferroin. The potential available in the titration of oxalate using the cerate ion is far higher than the oxidation potential of nitroferroin (1.75 volts *vs.* 1.25 volts). The oxidation potential of ferroin is, on the contrary, 1.06 volts. The titration error in the latter case would be expected to be much higher than in case nitroferroin were used. Premature fading of the indicator, nitroferroin, is consequently much less than in the case of ferroin. As a result, the required amount of indicator, nitroferroin, to produce a satisfactory color change can be destroyed by the use of 0.01 or 0.02 ml. of 0.001 *N* cerium as oxidant. The order of accuracy of such a titration is thus calculated to be  $\pm 0.05\%$ . The limit of accuracy above described was further tested by the use of weight burettes for sampling solutions for analysis. The same order of accuracy was obtained.

As a substitute for nitroferroin, the terpyridyl complex ion with ferrous iron may be employed. The color change in this case is from violet-red to colorless. Nitromethylferroin may also be used because of its correspondingly high oxidation potential (1.23 volts). Nitroferroin is preferred because it can be readily obtained and is less expensive.

Cholesterol values in the order of magnitude reported in Table V are found clinically in nephrotic conditions of nephritis, in certain anemias and in many other instances. That accurate calcium determinations in such plasma could not be performed by the technique of direct precipitation is indicated by the experiments reported in this paper with plasma containing between 565 mg. % and 900 mg. % cholesterol. If calcium is determined after direct precipitation in the plasma of patients with a cholesterol content of the indicated range, high calcium values might be erroneously reported. Although direct precipitation of calcium in the plasma might reveal correct values, there is no criterion for the exactness of the values obtained unless parallel cholesterol determinations are carried out. Consequently, ashing of the plasma prior to the precipitation of calcium is recommended.

### SUMMARY

A method for the quantitative determination of calcium in blood plasma is described. After ashing the sample with a  $\text{HClO}_4\text{-HNO}_3$  mixture, calcium is precipitated as  $\text{CaC}_2\text{O}_4$ ; the titration is carried out with ammonium hexanitratocerate in  $\text{HClO}_4$  solution and nitro-o-phenanthroline ferrous sulfate as indicator.

Some evidence is given which indicates that the presence of increased amounts of lipids interferes with the direct precipitation of calcium.

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# Glutamic Acid Decarboxylase of Higher Plants

## III. Enzymatic Determination of l(+)-Glutamic Acid

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### INTRODUCTION

The method described here utilizes as reagent an enzyme from higher plants which specifically decarboxylates l(+)-glutamic acid. The unnatural isomers of glutamic acid and other amino acids are not attacked and do not interfere with the rapid liberation of one mole of carbon dioxide from one mole of l(+)-glutamic acid. The procedure is therefore suitable for the determination of glutamic acid in protein hydrolyzates.

### EXPERIMENTAL

#### 1. Preparation of Glutamic Acid Decarboxylase

In a Waring Blendor 90 g. squash were mixed with 300 ml. water for 4 min. at room temperature. The material was then placed in a refrigerator for 1 hr. and later centrifuged for 15 min. (size 2 centrifuge, conical head, 4000 r.p.m.). The slightly turbid supernatant fluid (about 330–350 ml.) was rapidly frozen and dried *in vacuo* from the frozen state. Yellow squash yielded 3.5–4.5 g. dry powder, whereas white squash gave between 2.1 and 2.6 g. residue. The dried material was found to be very hygroscopic. It can be stored *in vacuo* over  $\text{CaCl}_2$  at room temperature without loss of enzymatic activity. Enzyme solutions were prepared by dissolving the dry powder from 90 g. squash in 30 ml. *m*/15 phosphate buffer, pH 5.75.

#### 2. Analytical Procedure

All experiments were performed with the Warburg apparatus at 37°C., using vessels with 2 side bulbs. The main vessel contained 4 ml. enzyme solution, the first side vessel received 0.5 ml. of the solution to be analyzed, adjusted to pH 6–7 and containing between 0.5 and 2.0 mg. glutamic acid. The second side bulb received 0.5 ml. 1.2 *N*  $\text{H}_2\text{SO}_4$ . An enzyme blank was set up, substituting 0.5 ml.  $\text{H}_2\text{O}$  for the solution containing glutamic acid. The efficiency of the enzyme preparation was

checked by substituting 0.5 ml. of a neutral 0.32% *l*(+)-glutamic acid solution for the material to be analyzed.

Acid hydrolyzates were neutralized with carbonate free NaOH. If the solution to be analyzed contained carbonate, or if carbonate was introduced while pH adjustments were made, a carbonate blank was set up, consisting of 4 ml. *m*/15 phosphate buffer, pH 5.75, in the main flask. 0.5 ml. 1.2 *N* H<sub>2</sub>SO<sub>4</sub> in the first side bulb and 0.5 ml. of the solution to be analyzed in the second side vessel. The amount of CO<sub>2</sub> liberated on addition of the unknown solution to the mixture of buffer and sulfuric acid is due to the carbonate content of the unknown and was subtracted from the amount of CO<sub>2</sub> liberated in the main experiment. The air in all vessels was replaced with nitrogen by passing a stream of the gas through the vessels for 3 min. in the usual manner.

The vessels attached to their manometers were immersed in the constant temperature bath and shaken for 20 min. to establish temperature equilibrium. Manometer readings were then taken and the contents of the first side bulb was tipped into the main vessel. The liberation of CO<sub>2</sub> started immediately and was practically complete after about 15–20 min. The reaction was allowed to continue for a total of 60 min. Readings were then taken and the contents of the second side bulb (sulfuric acid, except in the carbonate blank, see above) was tipped into the main vessel. The experiment was terminated with readings after an additional 10 min. of shaking. The readings obtained at that time remained constant on further incubation.

The amount of CO<sub>2</sub> produced by the enzyme blank was subtracted from the amount produced by the solution to be analyzed. A further correction, on a percentage basis, was made for overproduction of CO<sub>2</sub> observed with pure *l*(+)-glutamic acid, which, on the average, produced 102.6% of the calculated amount. The glutamic acid content of the unknown was then simply calculated by setting 152.3 mm.<sup>3</sup> CO<sub>2</sub> equivalent to 1 mg. glutamic acid.

For the determination of flask constants the solubility coefficient of 0.567 for CO<sub>2</sub> was used, which is slightly high but introduces only a negligible error. No attempts were made to read manometers better than to the next higher millimeter mark if the meniscus fell between two marks. The volumes calculated from pressure readings were rounded off to the nearest cubic millimeter.

### 3. Results with *l*(+)- and *dl*-Glutamic Acid

A total of 18 decarboxylations of pure *l*(+)-glutamic acid with 13 different squash preparations gave on the average 102.6% of the theoretical amount of CO<sub>2</sub> with maximal deviations of  $\pm 2.5\%$ . Fourteen of these results fell in the range  $103 \pm 1.6\%$  and 11 gave  $103.2 \pm 1\%$  of the calculated amounts of CO<sub>2</sub>.

A sample of *dl*-glutamic acid of high purity, obtained through the courtesy of Dr. D. Melnick, produced in three experiments 100.1, 98.9 and 100.5% (average 99.8%) of the amount of CO<sub>2</sub> expected from the *l*(+)-form present. A second sample, sold by Merck & Co. as *dl*-glutamic acid monohydrate was dried to constant weight *in vacuo*

over  $P_2O_5$  at  $61^\circ C$ . and lost 3.6% instead of 10.9% of water calculated to be present in the monohydrate. The dried sample produced 97.8 and 99.4% of the volume of  $CO_2$  expected from the amount of  $l(+)$ -form present. No decrease in the speed of decarboxylation was noticed in presence of the unnatural form of glutamic acid.

#### 4. *Decarboxylation of Glutamic Acid in Presence of other Amino Acids*

Mixtures of  $l(+)$ -glutamic acid with a variety of other amino acids were prepared and subjected to enzymatic decarboxylation. The results were corrected for the overproduction of  $CO_2$  observed with pure glutamic acid solutions by the same enzyme preparation. None of the added amino acids was decarboxylated and no interference with the decarboxylation of glutamic acid was experienced. A few typical examples are listed in the following tabulation:

Amino Acids in Mixture mg./100 ml.		Glutamic Acid Found mg./100 ml.	Recovery per cent
$l(+)$ -Glutamic acid	240	231.1	96.3
$l(+)$ -Aspartic acid	160		
$l(+)$ -Glutamic acid	320	322.6	100.8
$l(+)$ -Aspartic acid	120		
$l(+)$ -Glutamic acid	320	324.5	101.4
$l(+)$ -Lysine. $\times HCl$	160		
$l(+)$ -Glutamic acid	320	323.8; 331.8	101.2; 103.7
$l(-)$ -Arginine. $\times HCl$	800		
$l(-)$ -Leucine	800		
Glycine	600		
$l(-)$ -Cystine	40		
$l(-)$ -Tyrosine	40		
$l(+)$ -Histidine. $\times HCl$	20		

The maximal error of recovery in single experiments was 3.7% which is in the range of accuracy of the Warburg apparatus. Glutamic acid added to protein hydrolyzates was recovered practically quantitatively. Example: 5 ml. each of a neutralized protein hydrolyzate were pipetted into two test tubes. To one tube was added 5 ml. of water, to the second tube 5 ml. of a 0.32% solution of glutamic acid. The second tube contained, therefore, 16.0 mg. more glutamic acid than the

first tube. Both mixtures were decarboxylated enzymatically, using 0.5 ml. from each tube. The difference in  $\text{CO}_2$  formation between the two samples was found as 119 mm.<sup>3</sup>  $\text{CO}_2$  corresponding to 0.7814 mg. glutamic acid. The amount of glutamic acid added to the second tube was thus found as 15.63 mg., *i.e.*, 97.5% of the amount actually added.

### 5. Determination of Glutamic Acid in Protein Hydrolyzates

The glutamic acid content of a variety of protein hydrolyzates was determined enzymatically. Simultaneously, samples of the same

TABLE I  
*Glutamic Acid Content of Various Protein Hydrolyzates, Determined Enzymatically and by Chemical and Microbiological Methods*

Material	Assay Procedure	Glutamic Acid Content*	Average
Gluten-Hydrolyzate	chemical enzymatic	7.8	7.8
		8.05, 8.10, 8.20, 8.35	8.2
Basic Material FRL	microbiol. enzymatic	41.0	41.0
		39.41, 40.05	39.7
Casein-Hydrolyzate†	microbiol. enzymatic	19.5, 19.5, 19.6	19.5
		20.00, 20.44	20.2
Commercial Hydrolyzate no. 1	microbiol. chemical enzymatic	5.7, 6.4, 5.9, 5.0, 5.4	5.7
		5.4, 5.8, 5.8	5.7
		5.78, 5.80	5.8
Commercial Hydrolyzate no. 2	microbiol. enzymatic	1.5	1.5
		1.58, 1.58	1.6

\* The values for Basic Material FRL and for Casein are given as g. glutamic acid/100 g. protein. For the remaining hydrolyzates the values are g. glutamic acid/100 ml. solution.

† Not corrected for ash and moisture.

hydrolyzates were analyzed elsewhere for glutamic acid by chemical and microbiological methods. The results obtained with the method described here were in good agreement with the data obtained by other procedures, as shown in Table I.

## DISCUSSION

Glutamic acid is a prominent constituent of many proteins. In a recent tabulation of the composition of animal and plant proteins (1), 17 out of 19 contained more glutamic acid than any other amino acid. The industrial importance of glutamic acid can be seen from the fact that about 4,000,000 pounds of monosodium glutamate are produced and consumed annually in the U. S. A. (2). Glutamic acid seems to be an important link in the transformation of carbohydrates to proteins and participates in transaminations. Its presence in amino acid mixtures for intravenous feeding is considered to be the main reason for vomiting reactions (3) and the infusion of *l*(+)-glutamic acid alone (at rates of 2.5–5.0 mg./kg./min.) causes nausea and vomiting (4).

The availability of a simple and accurate procedure for the determination of *l*(+)-glutamic acid is, therefore, of interest to workers in many fields. A number of chemical procedures have been suggested, but are rather tedious. Attempts to find microbiological assay methods have been successful, but difficulties have been encountered, particularly if unknown growth effectors were introduced with crude hydrolyzates.

Gale (5) found that strain S.R. 12 (N. C. T. C. No. 6784) of *Cl. welchii* specifically decarboxylates only *l*(+)-glutamic acid and has used this organism for the determination of glutamic acid in protein hydrolyzates. The use of a pathogenic organism for routine analytical purposes is, however, not an attractive proposition. The plant enzyme used here is easily available and solutions with high activity are simple to prepare. As the enzyme preparations were found to be free of other amino acid decarboxylases, *l*(+)-glutamic acid can be determined directly in crude protein hydrolyzates without previous separation of glutamic acid from the mixture. Standardization of the assay procedure permits simultaneous analysis of a considerable number of proteins, limited only by the availability of a sufficient number of Warburg manometers, reaction vessels and thermostats.

## ACKNOWLEDGMENTS

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## SUMMARY

1. A method is described for the quantitative determination of *l*(+)-glutamic acid.
2. Crude protein hydrolyzates can be analyzed without removal of any constituents.
3. An enzyme solution (prepared from squash), which specifically decarboxylates the natural form of glutamic acid, is used in the assay procedure.
4. During the enzymatic reaction one mole of  $\text{CO}_2$  is liberated per mole glutamic acid.
5. The range of conventional Warburg manometers permits the use of samples containing between 0.5 and 2.0 mg. glutamic acid.

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# The Identification of Fructose

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A series of color reactions serve for the identification of fructose. Tests with resorcinol (Seliwanoff), diphenylamine (Ihl, Pechmann), indole and various derivatives (methylindole, tryptophan, carbazole) and ammonium molybdate (Pinoff) have been used in the form of several modifications. Due to the nonspecific character of the reagents mentioned, the presence of fructose can only be established with a certain degree of probability (1). It has been known for a long time that the different ketoses behave practically in the same way toward these color reagents (2). The different reducing properties of ketoses and aldoses with Ost's solution are also of limited value. Characterization by means of a typical crystalline derivative of fructose, which may be prepared in very small quantities must be preferred. Such a derivative is the methylphenylosazone. (3).

No doubt exists regarding the evidence of this reaction, first described in 1902. Emil Fischer (4) had shown that glucose does not form a methylphenylosazone when treated with methylphenylhydrazine acetate in the usual way. Neuberg then demonstrated that fructose behaves in an entirely different manner during this reaction (3). It forms, rapidly and in good yield, the methylphenylosazone which crystallizes well and melts at 158–160°C. This may also be derived from the glucosone (4), but since, according to present knowledge, osones do not occur in nature the methylphenylosazone reaction is of considerable specificity.

It has been reported since that under atypical conditions the methylphenylosazone might also be derived from glucose. For fructose Neuberg (3) has given the following directions: 1.8 g. fructose in 10 cc. water is heated for 5–10 minutes on the water bath with 4 g. methylphenylhydrazine and 4 cc. 50% acetic acid, adding just enough alcohol to obtain a clear solution. On scratching with a glass rod the osazone is precipitated. Ofner (5) did not obtain a methylphenylosazone either when he reacted glucose at 37–40°C. After 24 hours he claims 20% on inoculation, 12% without inoculation. On the other hand, fructose gave 58% typical methylphenylosazone in 24 hours. He, too, considers the test conclusive, provided the crystalline substance precipitates within 5 hours. Several later investigations are in sharp con-



tradition to Ofner's report. E.g., van der Haar (6) writes: "For the second time I found Neuberg's contention confirmed that methylphenylhydrazine is a reagent for ketoses in osazone formation." "Aldoses do not give any precipitate." In Tollens-Elsner (7) this statement is found: "It may further be recalled that only ketoses react with  $\alpha$ -methylphenylhydrazine to form osazones within a short time. This is a very valuable reaction." Percival and Percival (8) state: "The instructions of Ofner were followed, but the product was invariably the methylphenyl *hydrazone*; Neuberg's method, however, gave the methylphenyl*osazone*, melting point  $156^{\circ}\text{C}$ ." At the same time they found that the melting point reported by Ofner was more than  $50^{\circ}\text{C}$ . too low.

As described by different authors the presence of fructose has been conclusively established through the methylphenyl*osazone* in several cases. From the recent literature the surprising findings of Mann (9) may be cited. With the aid of the methylphenyl*osazone* he was able to identify the carbohydrate in semen as *d*-fructose.

It has now become possible to simplify the reaction considerably and, at the same time, increase its sensitivity.

If van der Haar (*l.c.*, p. 251) still believes 500 mg. to be the lower limit of detectability less than 1/100–1/200 of this quantity can easily be determined by the method described below.

In the original directions the free  $\alpha$ -methylphenylhydrazine was used in an acetic acid solution. However, the free base is an easily deteriorated, usually brownish material that can only be preserved in sealed ampoules and is difficult to handle. Furthermore, it is not readily obtainable everywhere. The methylphenylhydrazine sulfate,  $[(\text{CH}_3)(\text{C}_6\text{H}_5)\text{N} \cdot \text{NH}_2]_2\text{H}_2\text{SO}_4$ , on the other hand, is a stable white solid and can be bought in the pure state. It is at least as suitable as the free base for the preparation of the fructose methylphenyl*osazone*.

The sulfate crystallizes beautifully and the compound marketed by Eastman-Kodak can almost always be used directly. If the aqueous solution is yellow, recrystallization from ethyl alcohol and ether may be necessary. Stirring the product with a mixture of 4 parts ether and 1 part absolute alcohol often suffices to extract the yellowish colored impurity.

The osazone may be prepared in various ways:

(1) 4.1 g.  $\text{CH}_3 \cdot \text{COONa} \cdot 3\text{H}_2\text{O}$  are dissolved in 10 cc. warm water and 5.13 g. methylphenylhydrazine sulfate added. At room temperature the turbid liquid becomes clear on addition of 2 cc. glacial acetic acid. After cooling down to  $20$ – $25^{\circ}\text{C}$ ., the solution of 1.8 g. fructose in 2 cc. water is added. The liquid solidifies to a crystalline mass after standing

for one hour at ordinary temperature. Crystallization sets in after 25 minutes on inoculation.

If the glacial acetic acid is added before the sodium acetate (as described under 2 below) the cold solution becomes clear immediately.

Hofmann (10) is of the opinion that methylphenylhydrazine reacts less readily with ketoses than phenylhydrazine. In the case of fructose no distinct difference can be observed; the reaction starts more rapidly with methylphenylhydrazine but crystallization is completed faster with phenylhydrazine.

(2) 5.1 g. methylphenylhydrazine sulfate are dissolved in about 25 cc. water, adding 2 cc. glacial acetic acid and 4.1 g. crystalline sodium acetate. The clear cold solution is made up with water to a volume of 39 cc. and divided as follows:

- a. 6.5 cc.; To this 0.15 g. fructose in 1 cc. water is added.
- b. 13 cc.; To this 0.15 g. fructose and 0.15 g. glucose in 1 cc. water is added.
- c. 13 cc.; To this 0.15 g. fructose and 0.15 g. sorbose in 1 cc. water is added.
- d. 6.5 cc.; To this 0.15 g. glucose in 1 cc. water is added.

All the preparations are kept in closed test tubes. They are left at 22°C. for 10 minutes and then placed in the refrigerator at +4°C. After 20 hours *a*, *b* and *c* have become cloudy. On scraping they soon start to crystallize. After 48 hours in the refrigerator *a* had solidified completely, *b* and *c* partially, while *d* was still clear after 6 days at +4°C., no crystals separated out on further standing in the refrigerator for 2 days, even after scratching.

The crystals were filtered off by suction and in all cases proved to be typical reddish yellow fructose methylphenylosazone. Yields were in conformity with the amounts of fructose used. This series of experiments proves that neither glucose nor sorbose affects the reaction with fructose. (Again it was found that sorbose does not give a solid methylphenylosazone (3).) In the presence of sorbose resinous masses sometimes separate, which can be remedied by the addition of small amounts of alcohol.

The reaction mixtures have a pH of 4.55–4.58 and are well buffered. Since saccharose is stable at this pH the disaccharide does not react. (4.5 is the conventional pH for saccharose control in invertase investigations.) The crystallization of fructose methylphenylosazone is not affected by the presence of saccharose.

(3) 0.9 g. fructose in 1 cc. water are added to a solution of 2.55 g. methylphenylhydrazine sulfate, 1 cc. glacial acetic acid and 2.05 g.  $\text{CH}_3\cdot\text{COONa}\cdot 3\text{H}_2\text{O}$  in 19 cc. water.

- a. 2 cc. of this solution, containing 0.09 g. fructose, are placed in the refrigerator at  $+4^\circ\text{C}$ .
- b. 2 cc., containing 0.09 g. fructose are left at  $28-30^\circ\text{C}$ .
- c. 0.5 cc., containing 0.0225 g. fructose are placed in the refrigerator.
- d. 0.5 cc., containing 0.0225 g. fructose are left at  $28-30^\circ\text{C}$ .

All the preparations were kept in small closed test tubes. Those left at room temperature were completely solidified after 70 min. After 20



FIG. 1

Fructose Methylphenylosazone (Raw)

minutes the solution had become turbid, after 30 minutes crystallization had set in. After 30 hours the preparations left in the refrigerator had also solidified to a crystalline mass.

The test is successful with 0.1 and 0.05 cc. of the original solution, *i.e.*, with 0.0045 and 0.00225 g. fructose, respectively. If the tiniest drop of mixture *d* is placed on a microscope slide with single concavity by means of a capillary and then scratched with a glass thread, preventing evaporation by means of a cover glass, beautiful yellow needles, in bundles or radially arranged, are obtained at room temperature

after 40 minutes or even earlier. (See photomicrograph 1.) *Thus, the limits of identification are the limits of the microtechnique.*

In ordinary macro determinations, *i.e.*, with 0.9 g. fructose, it can be observed that the solution rapidly attains a reddish yellow coloration, while the otherwise identical preparations with glucose, which do not give an osazone, remain pale yellow.

The yields have been determined in the macro procedure with 0.9–1.8 g. fructose. They were found to be 64–80% of the theoretical, *i.e.*, well within the limits of normal osazone yields. In accordance with

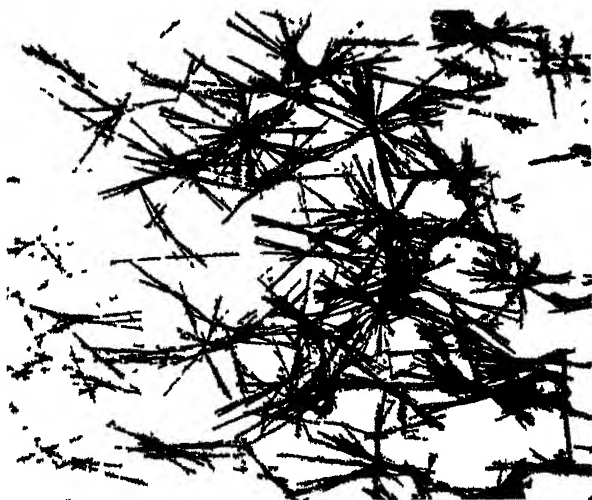


Fig. 2

Fructose Methylphenylosazone, Recrystallized from Ethyl Acetate

previous observations decomposition takes place at 158–160°C. Recrystallization from ethyl acetate or aqueous methyl cellosolve or dioxane yields magnificent long needles. (See photomicrograph 2.)

We are indebted to Miss Mary Schuster of the Interchemical Corp., N. Y., for the photomicrographs.

*Summary:* Under the most varied conditions formation of the methyl phenylosazone proves to be a specific reaction for fructose. It can be carried out in the presence of glucose, saccharose and sorbose and as a micro reaction. It allows clearcut differentiation between fructose and glucose. The methylphenylosazone from fructose forms even at + 4°

(in the refrigerator). It separates in well-developed characteristic crystals and decomposes at 158–160°C. Directions are given for the use of the stable methylphenylhydrazine sulfate, easy to handle and obtainable in a pure state, instead of the free methylphenylhydrazine, which readily decomposes and is disagreeable to handle.

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# Quantitative Formation of Osazones

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Since Emil Fischer (1) discovered the osazones 62 years ago attempts have frequently been made to render this important reaction quantitative. An extensive literature regarding the phenylosazones exists, from which the following facts may be cited.

Maquenne (2) stated that, under standard conditions, the isolation of phenylosazones from hexoses is possible in yields of 12–41%. Later experiments of Knecht and Thompson (3) gave the highest yield obtained thus far, *i.e.*, 84% in the case of glucose. This was realized when the reaction took place in 20% acetic acid solution in the presence of aniline and ammonium acetate. The optimum pH would be about 4.5. A higher concentration of acetic acid lowered the yield due to secondary alterations of the phenylhydrazine (4). In one case Gerard and Sherman (5) also reported a yield of 84%, while Butler and Cretcher (6) obtained yields of 63–67%. Fischer and Baer (7) obtained 80.5% yield with sorbose and 72.8% with fructose, and Glassmann and Rochwarger-Walbe (8) got a maximum yield of 70% with glucose.

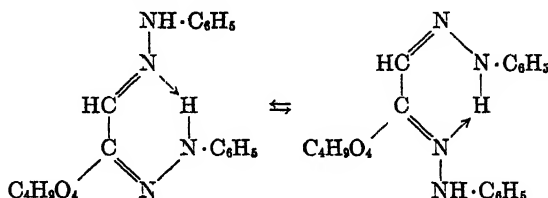
The interesting fact that acetic acid acts as a catalyst and that aniline increases the yield was found empirically at an early date, as will be seen. Weygand (9) has proposed a new explanation for the mechanism of the osazone reaction. An Amadori-rearrangement occurs, *i.e.*, every aldose first forms an *N*-glycoside with primary aromatic amines (aniline and its homologues) and this rearranges to a substituted  $\alpha$ -amino ketose, an aryl isoglucosamine. For both the formation of the *N*-glycoside—Cameron (10)—and for its transformation to the aryl isoglucosamine—Weygand (9)—the presence of acetic acid is necessary. As simple aromatic amines form aryl glucosamines with aldoses faster than phenylhydrazine, while the aryl isoglucosamines react very rapidly with phenylhydrazine, the yield is increased by condensation of an arylamine with the sugar before treatment with the solution of phenylhydrazine acetate. Even with this procedure,\* however, Weygand obtained maximum yields of no more than 72–80%.

According to L. F. Fieser and M. Fieser (11), Weygand's explanation of the formation of osazones is unsatisfactory. They find that it fails to answer the question of

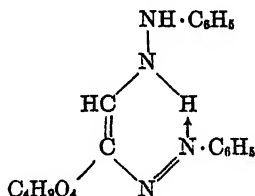
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\* The first phase is practically a fusion of the sugar with the aromatic amine in presence of some acetic acid. Differing from the usual conditions of the osazone reaction, this procedure is carried out in a medium which is nearly water-free.

why the reaction stops with introduction of only two phenylhydrazine rests. The possibility of stabilization of the osazone by formation of a tautomeric chelate ring structure should be considered:



Effects of resonance according to the formula



might also be taken into account.

As far as we know there is only one publication regarding the yields of osazones formed with substituted phenylhydrazines. Knecht and Thompson (3) obtained a maximum yield of 67%, as calculated from their data, when forming the *p*-nitrophenylosazone from glucose.

Only in the case of trioses is the osazone reaction quantitative: from dihydroxyacetone and from glyceraldehyde the corresponding bishydrazones of methylglyoxal can be obtained quantitatively (12) on treatment with *p*-nitrophenylhydrazine in acetic acid or with 2,4-dinitrophenylhydrazine in hydrochloric acid solution. It is evident that in this reaction, which has become the routine method for determination of trioses and their esters, the result is due to secondary formation of a representative of the 1,2-dicarbonyl compounds, which can react quantitatively with the nitrophenylhydrazines. Neuberg and Strauss (13) have shown that this is a general property of such compounds.

From its formula the osazone reaction may be described as the formation of a bishydrazone after oxidation of the aldose or ketose to the osone step, *i.e.*, to a 1,2-dicarbonyl compound. Brady (14) makes the strange statement that  $\alpha$ -hydroxyaldehydes and  $\alpha$ -ketones react with 2,4-dinitrophenylhydrazine with great difficulty and sugars do not react at all. Our own experiences (13) with lactic acid aldehyde

and with acetol are contrary to this. On the basis of these observations we attempted to find out whether sugars do not also give quantitative yields of 2,4-dinitrosoazones with 2,4-dinitrophenylhydrazine.\*) This end was attained with glycolaldehyde, the hexoses and *d*-glycero-*d*-guloheptose.

The DN is applied in a strong mineral acid solution and the heating must be prolonged to complete the reaction. Consequently, di- and polysaccharides are hydrolyzed and pentoses partially transformed to furfural. The method cannot be used, therefore, in its present form with these carbohydrates. Under the conditions of the DNO reaction the transformation of pentoses into furfural is not quantitative so that precipitation of furfuralhydrazone cannot be used as an indirect method for the determination of pentoses. It thus differs from the quantitative transformation of trioses into methylglyoxal-bis-2,4-dinitrohydrazone. [In the method using distillation of furfural and methylfurfural with HCl, on the other hand, DN may be used for quantitative determination of pentoses and methylpentoses, as reported by Simon (15).]

There are slight differences in the behavior of the epimeric aldoses and the coordinated ketoses. The reaction starts faster with the latter and is practically quantitative with, *e.g.*, fructose, glucose and mannose.

### EXPERIMENTAL

The reaction is usually carried out as follows: 1 mol of the sugar is dissolved in water and a hot solution—filtered if necessary—† of 3 mols of DN in 2 *N* HCl (60 times the weight of the reagent) is added, as well as 1/100 vol. ethanol.‡ The mixture is heated under a reflux in the boiling water-bath for 12–24 hours. On shaking the precipitate of DNO flocculates and the supernatant liquid becomes clear. If no further turbidity appears on continued heating, the reaction can be regarded as complete. The DNO is filtered from the warm (55°C) liquid on a sintered glass crucible washed with cold dilute HCl, then with water and dried in a vacuum desiccator. The filtration is carried through with ease. All DNO osazones have a reddish brown or red coloration.

We have already shown that, in the case of the simple dicarbonyl compounds, many hours heating is necessary to complete the reaction. This applies even more for the reaction with sugars.

\* The following abbreviations will be used henceforth:

DN 2,4-dinitrophenylhydrazine,

DNO 2,4-dinitrophenylosazone.

† The DN supplied by Eastman Kodak Co. is usually perfect. If it should not dissolve in the 60-fold amount of hot 2 *N* HCl it may be recrystallized from ethyl acetate.

‡ The addition of ethanol is not absolutely necessary but it stabilizes the reagent.



It should be stated that the DNO substances are found to be crystalline. (The same is true of the *p*-nitrophenylosazones of the sugars, which up to now have been isolated as amorphous powders only (16); but when prepared in HCl solution instead of acetic acid they are found to be micro-crystalline directly.)

Crystals of the nitrophenylosazones can be obtained from dilute dioxane, tetrahydrofurfuryl alcohol or from methylcellosolve or ethyl acetate.

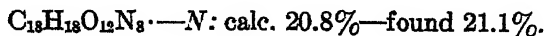
*Glycolaldehyde-2,4-dinitrophenylosazone*

For the preparation of this substance, crystalline glycolaldehyde, made according to Collatz and I. S. Neuberg (17), may be used, or else glycolaldehyde-diethylacetal, which is entirely hydrolyzed under the conditions of the reaction. Three-tenths g. of the hydroxylaldehyde or 0.67 g. of its acetal are added to a hot solution of 3 g. DN in 180 ml. 2 *N* HCl. A yellow precipitate of the hydrazone is first formed but, on further heating, this soon changes to the orange DNO. Heating was continued for 12 hours. The yields were 2.02 g. and 1.99 g. instead of the theoretical 2.07 g., *i.e.*, 96.6–95%. This DNO was identical with the compound previously prepared from glyoxal (18).

*l-Sorbose-2,4-dinitrophenylosazone*

Ten ml. of an aqueous solution of *l*-sorbose, containing 0.999 g. of the sugar, were added to a hot solution of 0.4 g. DN in 25 ml. of 2 *N* HCl with 0.4 ml. ethanol and the mixture heated on the waterbath for 20 hours. The osazone was treated as described above. It separates as a solid mass and is practically pure. As opposed to the phenylosazone of sorbose it is never gelatinous but micro-crystalline. The yield was 0.292 g., *i.e.*, 98% of the theoretical amount. The DNO is insoluble in water and only slightly soluble in ethyl alcohol, methyl alcohol and ether, soluble in glycol monomethyl ether and pyridine.

Somewhat larger crystals, generally associated as clusters, can be obtained by the following procedure: Water is added to the hot solution in tetrahydrofurfuryl alcohol until it becomes turbid. The mixture is then reheated until clear, filtered and cooled very slowly. The substance decomposes at 230°C.



*d-Galactose-2,4-dinitrophenylosazone*

This osazone, also unknown up to the present, has been prepared in the manner described above. Heating was continued for 24 hours. The yield was 97% of the theoretical amount.

The product is insoluble in water, difficultly soluble in petrol ether, methanol, ethanol, ethyl ether, chloroform and benzene; it is soluble in nitrobenzene and ethyl acetate, very easily soluble in pyridine, from which it can be precipitated with sulfuric acid, and in dioxane and tetrahydrofurfuryl alcohol, from which it can be separated by addition of water. The substance is recrystallized from ethyl acetate or methylcellosolve. It gradually decomposes above 185°C.

$C_{18}H_{18}O_{12}N_8$ .—*N*: calc. 20.8%—found 20.95%.

Like all DNO-products, the sorbose and galactose derivatives dissolve in an alcoholic solution of NaOH with the violet color characteristic of these dicarbonyl derivatives.

*d-Glucose-2,4-dinitrophenylosazone*

This compound has been prepared from *d*-glucose, *d*-fructose and *d*-mannose, heating for 20, 17, and 24 hours, respectively. The yields were 98.5% for *d*-glucose, 99% for *d*-fructose and 97% for *d*-mannose.

Glaser and Zuckermann (19) have obtained this derivative from glucose, but they did not state the yield. Under no circumstances could it have been quantitative, however, as the authors used incorrect values in their preparation. They only added 0.8 g. DN to 1 g. glucose, instead of the 3.33 g. of reagent necessary for complete reaction.

In this connection it may be stated that the formation of a DNO with DN requires 3 mol DN, because here, too, one mol of the hydrazine acts as a dehydrating agent on carbon-atoms 1 or 2. This is established by the fact that 1 mol of DN is split into 1 mol of  $NH_3$  and 1 mol of 2,4-dinitraniline. The presence of the latter can be proved. This mechanism of reaction is not quite obvious. Dehydration to the osone step could also have been brought about by the nitro groups of the DN. However, 2,4-dinitraniline is formed and has been isolated from the reaction mixture.

In the described procedure the glucose-DNO separates at once in short needles. In addition to dioxane, cellosolve and tetrahydrofurfuryl alcohol it may also be recrystallized from excess ethanol, *n*-propanol or

ethyl acetate. From this latter solvent well-developed long needles are obtained. The glucose-DNO has been described as an amorphous powder, yielding crystals from pyridine. However, these crystals are not the pure DNO, but a pyridine double compound. The same objections also apply to the statements (19) regarding the DNO of  $\alpha$ -glucoheptose. The crystals separating from pyridine may be washed with ligroin on the suction filter. After drying to constant weight over  $\text{H}_2\text{SO}_4$  the remaining substance no longer has an odor of pyridine, but yields pyridine on heating with strong  $\text{NaOH}$ , which can be established in the distillate by its odor, with  $\text{HgCl}_2$  and Vongerichten's reaction. The glucose-DNO can be precipitated from a warm pyridine solution with dil.  $\text{H}_2\text{SO}_4$ ; after thorough washing it is found to be free from pyridine but the separated DNO is not distinctly crystalline.

*d-Glycero-d-guloaldoheptose-2,4-dinitrophenylosazone*

This substance can be prepared in 98–100% of the theoretical yield, if 1.5 g. DN, dissolved in 90 ml. 2 *N*  $\text{HCl}$  and 1 ml. ethanol is allowed to act on 0.53 g. of the heptose in the waterbath for 16 hours, *i.e.*, 3 mol to 1 mol instead of the incorrect ratio given for heptose in the literature (19). No yield has been stated but the amount of DN used was almost 50% too low.

The heptose-DNO is insoluble in water and soluble in dioxane, ethyl acetate and tetrahydrofurfuryl alcohol; from its solution in nitrobenzene the substance can be precipitated with petrol ether. The osazone can be recrystallized from ethyl acetate. It decomposes at 232–233°C.

$\text{C}_{19}\text{H}_{20}\text{O}_{12}\text{N}_2$ :—*N*:calc. 19.7%—found 19.9%.

Some experiments made with glucosamine hydrochloride did not give satisfactory results. The glucosamine reacts rather slowly and incompletely with DN.

As previously mentioned, di- and polysaccharides are hydrolyzed under the conditions of the DN-reaction. Special experiments showed that maltose, turanose, saccharose, raffinose and melezitose form hexosazones. The yield was 95–99% of the monosaccharides contained in the sugars. In every case the reaction starts rapidly but requires 18–24 hours for completion when the sugars are heated in the boiling waterbath with 3 mol DN per hexose-unit in hydrochloric acid solution. In the case of lactose the reaction starts somewhat slower, consistent with

the experience (20) that the hydrolysis of this disaccharide only occurs after some delay. The yield of hexosazones also amounted to only 83–85% of the theoretical. On the other hand almost quantitative yields were obtained with  $\alpha$ -methyl-*d*-glucoside and 2-hydroxybenzyl-alcohol- $\beta$ -glucoside (salicin).\*

A considerable amount of osazone can thus be obtained from both reducing and non-reducing sugars by a one-step process. For every 1.8 mg. hexose-unit 5.38 mg. DNO are formed, *i.e.*, approximately a 3-fold amount. In certain cases this fact is analytically advantageous. Since the DNO reaction can thus be carried out within  $\gamma$ -limits and—as has been shown above—is practically quantitative, the micro determination we described a short time ago (21) can be employed.

#### THE DNO-REACTION AS APPLIED TO SOME PHYSIOLOGICAL MATERIALS

*Urine and Blood.* There is no difficulty involved in the quantitative separation of glucose as DNO from diabetic urine. If it contains more than 0.2% the urine can be utilized directly. The necessary amount of DN is calculated from the volumetrically or polarimetrically determined sugar content. The DNO obtained after heating for 20 hours is superficially dried and then washed with petrol ether to remove resinous admixtures. The weight conforms to the sugar content found by other methods.

For smaller amounts of sugar previous purification of the urine is recommended. This is effected by the well known lead acetate method, excess reagent being removed later with  $\text{Na}_2\text{SO}_4$ . The subsequent procedure is exactly as described. Normal urine also contains carbohydrate-like substances, in particular glucuronides. The amount of DNO formed from them is slight and quantitatively insignificant. Especially after purification with lead acetate, not more than a few small flocks separate out. The fact that they include derivatives of uronic acids is seen by dissolving in sodium carbonate with a violet coloration, the glucose-DNO remaining insoluble in  $\text{Na}_2\text{CO}_3$ .

Blood sugar can also be separated as DNO. Deproteinization is preferably accomplished with perchloric acid, as recently described (21). The amount of blood sugar deduced from the weight of the DNO agrees with the result of the volumetric method by Hagedorn-Jensen. (Sugar phosphates present in the blood are included in the determination, since hydrolysis occurs on heating in hydrochloric acid solution.)

\* Resinous condensation products derived from the salicylic alcohol split off may be removed from the DNO by means of petrol ether.

*Plant Materials.* The procedure may be adapted to plant materials. It is of value in cases where practically quantitative separation of carbohydrates as a solid derivative of which a  $\gamma$ -portion is sufficient for a subsequent micro determination is desirable.

#### ACKNOWLEDGMENTS

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#### SUMMARY

All former attempts to make the osazone reaction quantitative have failed. By means of 2,4-dinitrophenylhydrazine in HCl-solution the quantitative precipitation of glycolaldehyde, *d*-glucose, *d*-mannose, *d*-fructose, *d*-galactose, *l*-sorbose and *d*-glycero-*d*-guloaldoheptose as 2,4-dinitrophenylosazones is possible. The method is given in full and the properties of the different substances are described. From di- and polysaccharides, the hexosazones of the monosaccharides forming these sugars can also be prepared quantitatively.

The method can be applied to sugar-containing materials from physiological sources. The separated 2,4-dinitrophenylosazones can be determined colorimetrically within  $\gamma$  limits.

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# The Effect of Certain Azo Dyes upon the Storage of Riboflavin in the Liver \*

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## INTRODUCTION

A connection between riboflavin and the development of hepatic tumors due to azo dyes was first indicated by Kensler, Sugiura and Rhoads (1), who showed that the concentration of this vitamin in rat liver decreased when *p*-dimethylaminoazobenzene was fed in a crude diet of rice and carrot. Furthermore, the combination of riboflavin and casein added to the crude diet retarded tumor development markedly (2). Experiments at the University of Wisconsin suggest that riboflavin is the important component in the combination, for the vitamin greatly delayed the formation of hepatic tumors when added to synthetic diets containing *p*-dimethylaminoazobenzene (3). Apparently casein contributes to the efficient utilization of riboflavin; Sarett, Perlzweig and others have shown that dietary protein increases the capacity of the liver to store this vitamin (4, 5, 6).

In the original experiments of Kensler *et al.* (1, 2) no attempt was made to equalize the consumption of food between the groups of rats fed the azo dye or the control diet, and therefore the intakes of protein and riboflavin in the two groups were probably unequal. Rats fed *p*-dimethylaminoazobenzene consume only 50-75% as much food as those on corresponding diets free from the compound (7). Hence, data on the specific effect of the azo dye on hepatic riboflavin appeared desirable. The present study also includes data on related azo dyes that are either more or less carcinogenic than *p*-dimethylamino-

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azobenzene itself (8). Riboflavin counteracts the carcinogenicity of at least three of them to some extent: *p*-monomethylaminoazobenzene (9), *o*'-methyl-*p*-dimethylaminoazobenzene (10), and *m*'-methyl-*p*-dimethylaminoazobenzene (10). It appeared possible that the effect of a given azo dye on hepatic riboflavin might parallel its potency in inducing liver tumors.

## METHODS

Both young and adult rats were fed semi-synthetic diets (Table I) containing the various azo dyes. The experiments were performed in series, in each of which two control groups received the basal ration free from dye fed either *ad libitum* or restricted in amount to that consumed by a group receiving *p*-dimethylaminoazo-

TABLE I  
*Composition of Diets*  
g./kg.

Diet No	I	II	III	IV
Cerelose*	790	670	790	670
Casein (purified)	120	240	120	240
Salts mixture	40	40	40	40
Corn oil	50	50	50	50
Riboflavin	.004	.004	.002	.002

Each kg. of diet contained 3.0 mg. of thiamine chloride, 2.5 mg. of pyridoxine hydrochloride, 30 mg. of choline chloride, and 7.0 mg. of calcium pantothenate.

\* Pure glucose monohydrate obtained from Corn Products Refining Co.

benzene or *m*'-methyl-*p*-dimethylaminoazobenzene. The concentrations of dye were varied from series to series and when comparisons between compounds were made, this was done on the molar basis. The dyes fed included *m*'-methyl-*p*-dimethylaminoazobenzene, *p*-dimethylaminoazobenzene, *p*-monomethylaminoazobenzene, *o*'-methyl-*p*-dimethylaminoazobenzene, *p*'-methyl-*p*-dimethylaminoazobenzene, aminoazotoluene, azobenzene and *p*-aminoazobenzene. The compounds were either synthesized in this laboratory or were samples used in previous studies (8).

Animals fed *ad libitum* were kept in groups of 3 to 6 in screen bottom cages. Those on restricted food intakes were kept in single cages. At the end of the feeding time, which was either 3 or 6 weeks, the animals were killed by decapitation. The livers were removed, weighed, homogenized in a Waring blender with 0.1 N H<sub>2</sub>SO<sub>4</sub>, and autoclaved for 15 minutes at 15 lbs. pressure. Riboflavin was determined fluorometrically according to the method of Connor and Straub (11) with certain modifications suggested by Andrews (12). A similar technique was employed for the analysis of tissues other than the liver. Values were reported as  $\gamma$  of riboflavin/g. of fresh liver and also as  $\gamma$ /entire organ.

## RESULTS

The addition of *p*-dimethylaminoazobenzene (DAB) to the diet invariably lowered the riboflavin content of the liver (Table II). This is in line with the original observation of Kensler *et al.* (1, 2) and was true whether the basal diet contained 12 or 24% of casein (diets I and

TABLE II  
*Relative Effect of Azo Dyes and Caloric Restriction on Liver Riboflavin*

Diet and dye fed	No. animals	Init. wt.	Growth increment	Average daily food intake	Liver riboflavin		
					A <sub>1</sub>	Range	Total
		<i>g</i>	<i>g 'mL./rat</i>	<i>g /rat</i>	<i>γ/g</i>	<i>γ/g</i>	<i>γ</i>
I Control, <i>ad libitum</i>	3	49	8	7.8	15	12.0-19.0	61
I .06% DAB	3	41	3	5.3	13	11.7-14.7	37
II Control <i>ad libitum</i>	3	47	20	8.6	21	19-22.5	117
II Control restricted	3	55	11	7.8	23	21-24	90
II .06% DAB	3	44	11	7.2	15	13.7-16.5	64
II .064% <i>m'</i> -MeDAB	3	43	8	6.5	13.7	12.7-15.5	53
II .053% AAT	3	39	16	8.5	17.3	17-18	100
*III Control <i>ad libitum</i>	3	253	1	12.8	18.1	17-19.6	146
*III Control restricted	3	237	.0	11.2	17.7	15.5-19.9	158
*III .06% DAB	3	241	-1	11.2	13.8	12.5-15.5	111
*III .064% <i>m'</i> -MeDAB	3	264	-8	9.5	9.8	8-11	83†
III Control <i>ad libitum</i>	2	285	-7	13	16.8	16-17.5	165
III .060% DAB	3	259	-6.7	11.9	14.5	13.6-15.5	136
III Control restricted	3	250	+1.7	11.9	20.8	19-21.5	163
III .064 <i>m'</i> -MeDAB	3	277	-24	7.9	11.6	10-13	82
III Control restricted	4	197	-2	7.9	18.4	17-20	125
III Control <i>ad libitum</i>	4	202	6	11	20.2	19.2-21.5	161
III .09% DAB	4	197	-7	8.0	14.9	14.7-15	111
III Control, restricted	4	197	-2	8.0	18.4	17-20	125
III .096% <i>m'</i> -MeDAB	3	214	-15	6.0	13.7	10-17	71
III Control, restricted	4	209	-8	6.0	22.7	21-25	130

\* Feeding time 6 weeks.

† Cirrhosis at 6 weeks.

DAB = *p*-Dimethylaminoazobenzene.

*m'*-MeDAB = *m'*-methyl-*p*-dimethylaminoazobenzene.

AAT = Aminoazotoluene.

2), whether the riboflavin content of the diet was 2 or 4 mg./kg. (diets 3 and 4 versus 1 and 2), whether the rats were weanlings or adults of either sex, whether the experiment was conducted for 3 or 6 weeks, or whether the concentration of the dye in the diet was 0.06% or 0.09% of *p*-dimethylaminoazobenzene.

In a typical experiment the  $\gamma$  of riboflavin/g. of liver were 14.5 for the group fed 0.06% *p*-dimethylaminoazobenzene, 16.8 on the control diet fed *ad libitum*, and 20.8 in the group fed the control diet restricted in amount to that consumed by animals receiving *p*-dimethylaminoazobenzene. The total amounts of riboflavin per liver were 136, 165 and 163  $\gamma$ , respectively. Table III indicates that the concentrations of

TABLE III  
*The Effect of Azo Dyes on the Riboflavin Content of Various Tissues*

Diet and dye fed	No. animals	Initial weight	Growth increment	Average daily food intake	Riboflavin				
					Liver	Kidney	Heart	Muscle	
					Total				
I 0.06% DAB	5	64	4	7.5	12.5	50	21	14	2.5
I Control, restricted	5	64	6.4	7.5	170	62	21	12	2.3
II 0.06% DAB	5	65	11	7.7	17.6	94	22	13	2.3
II Control, restricted	5	66	13	7.7	24.9	99	22	14	2.3
II 0.064% <i>m'</i> -MeDAB	5	64	7.5	7.7	16.5	74	23	11	2.6

Feeding time 3 weeks.

DAB = *p*-dimethylaminoazobenzene.

*m'*-MeDAB = *m'*-methyl-*p*-dimethylaminoazobenzene

the vitamin in the kidney, heart and skeletal muscles were not altered under experimental conditions in which the dye caused the riboflavin content of the liver to be reduced from 17 to 12.5  $\gamma$ /g. of liver in one series, and from 24.9 to 17.6 in another.

*m'*-Methyl-*p*-dimethylaminoazobenzene, which is a more potent carcinogen than *p*-dimethylaminoazobenzene, also proved to be consistently more effective in lowering the riboflavin content of the liver. This was true under all of the various experimental conditions employed. *m'*-Methyl-*p*-dimethylaminoazobenzene is somewhat more

toxic than *p*-dimethylaminoazobenzene (8, 13) and, in the present experiments, the consumption of food containing this dye was invariably less than that of diets containing *p*-dimethylaminoazobenzene. It could be demonstrated, however, that the additional lowering of the riboflavin content of the liver was not due to the more severe restriction of food intake. For example, in one series in which .09% of *p*-dimethylaminoazobenzene or the molar equivalent of the *m'*-methyl derivative was fed for 3 weeks to adult rats, the group receiving *p*-dimethylaminoazobenzene consumed 8 g. of food per day whereas those on *m'*-methyl-*p*-dimethylaminoazobenzene consumed only 6 g. (Table II). Rats on the control diet fed *ad libitum* ate 11 g. of food per day. Control animals restricted to 8 g. per day contained 18.4  $\gamma$  of riboflavin/g. of liver or 125  $\gamma$  per organ. Those restricted more severely to 6 g. per day contained 22.7  $\gamma$ /g. of liver or 130  $\gamma$ /organ. The amounts of riboflavin remaining in the livers of rats receiving the dye were 111  $\gamma$ /liver in the animals receiving *p*-dimethylaminoazobenzene, and only 71  $\gamma$  in the livers of rats receiving *m'*-methyl-*p*-dimethylaminoazobenzene.

*p*-Monomethylaminoazobenzene, another potent carcinogen (8, 9), also proved to be effective in lowering the riboflavin content of the liver (Table IV). When .084% of the monomethyl derivative was fed to adult rats, almost identical amounts of riboflavin were found in the liver as when a molar equivalent of *p*-dimethylaminoazobenzene was fed: 106 and 108  $\gamma$ , respectively. The amounts in the two control groups in this series were 155 and 129  $\gamma$ /organ respectively. When the monomethyl compound was fed to young rats (Table IV), somewhat larger amounts of hepatic riboflavin were found than in the corresponding group receiving *p*-dimethylaminoazobenzene; but the amount was less than that in groups receiving any of the other azo compounds fed except *p*-dimethylaminoazobenzene and *m'*-methyl-*p*-dimethylaminoazobenzene. Since *p*-monomethylaminoazobenzene and *p*-dimethylaminoazobenzene are interconvertible in the liver (14), some similarity between the action of the two on hepatic riboflavin might be expected.

The other azo dyes, *o'*-methyl-*p*-dimethylaminoazobenzene, *p'*-methyl-*p*-dimethylaminoazobenzene, aminoazobenzene, azobenzene and aminoazotoluene, had little, if any, effect on hepatic riboflavin, with the possible exception of *o'*-methyl-*p*-dimethylaminoazobenzene which appeared to lower the riboflavin content of older animals when .096% of the dye was fed. *o'*-Methyl-*p*-dimethylaminoazobenzene is

very definitely carcinogenic, being about two-thirds as active as *p*-dimethylaminoazobenzene (8, 10). The variations in the effect of the other dyes on the storage of the vitamin are probably without significance. None of them exerted any great effect on hepatic riboflavin

TABLE IV  
*The Relative Effect of Azo Dyes on Liver Riboflavin*

Diet and dye fed	No. animals	Initial weight	Growth increment	Average daily food intake	Liver riboflavin		
					Average	Range	Total
III Control <i>ad lib.</i>	6	189	11	12.9	17.1	(15-19)	155
III Control restricted	6	196	-10	5.5	24.1	(20-27)	129
III .096% <i>m'</i> -methyl- <i>p</i> -dimethylaminoazobenzene	3	209	-12	5.5	10.6	(10-11)	51
III .09% <i>p</i> -dimethylaminoazobenzene	5	217	-8	8.6	13.7	(12-15)	108
III .084% <i>p</i> -monomethylaminoazobenzene	5	207	-10	7.3	15.5	(12-17)	106
III .096% <i>o'</i> -methyl- <i>p</i> -dimethylaminoazobenzene	6	206	-10	8.9	16.1	(14-19)	121
III .096% <i>p'</i> -methyl- <i>p</i> -dimethylaminoazobenzene	4	207	-3	10.8	16.3	(15-16.8)	125
III .09% 2-amino-5-azotoluene	4	216	-6	10.0	17.4	(15.5-20)	136
III .074% azobenzene	5	206	-8	7.9	17.9	(15-19)	128
III .082% <i>p</i> -aminoazobenzene	5	207	-4	9.1	19.4	(17-21)	134
IV Control <i>ad lib.</i>	5	71	17	9.4	14.0	(12.6-15)	90
IV Control restricted	5	67	11	6.3	17.9	(15.3-19.5)	80
IV .064% <i>m'</i> -methyl- <i>p</i> -dimethylaminoazobenzene	5	60	6	6.3	11.0	(10-11.8)	41
IV .06% <i>p</i> -dimethylaminoazobenzene	5	69		7.2	11.6	(11-12)	57
IV .056% <i>p</i> -monomethylaminoazobenzene	4	60		6.9	15.3	(13-17)	74
IV .064% <i>o'</i> -methyl- <i>p</i> -dimethylaminoazobenzene	5	62	13	7.5	14.4	(10-18)	
IV .064% <i>p'</i> -methyl- <i>p</i> -dimethylaminoazobenzene	5	71	16	8.4	11.3	(9-13)	92
IV .06% aminoazotoluene	5	64	14	9.0	15.0	(13.5-18)	97
IV .049% azobenzene	5	69	8	7.5	17.9	(16.6-19.5)	87
IV .053% <i>p</i> -aminoazobenzene	5	71	18	9.0	18.3	(17-23)	99

Feeding time 3 weeks.

under the conditions of these experiments, and such irregularities as appeared may well have been associated with differences in food intake and in liver size. An apparent exception to the general rule is that animals receiving azobenzene, which is non-carcinogenic (15), consumed considerably less food than those on aminoazotoluene, a definite carcinogen for both mice and rats (15). In general, however, the present data indicate rather clearly that the most potent carcinogens are very effective in decreasing the riboflavin content of the liver, moderate carcinogens are intermediate in this respect, whereas none of 4 inactive or very weak carcinogenic dyes had any effect on riboflavin storage under comparable conditions. It is, therefore, tempting to postulate that the retention of riboflavin in the liver is in some way connected with resistance to reactions by which liver tumors arise, although the vitamin may also be involved in the detoxification of specific azo dyes. But in view of the divergent results obtained with the different compounds tested, it does not appear that the azo linkage *per se* is the critical factor in decreasing hepatic riboflavin.

Incidentally, the present data confirm the observation (6) that an increase in the casein content of the diet increases the riboflavin content of the liver (Tables II and III). Young rats fed the control diet *ad libitum* contained 15  $\gamma$  of riboflavin/g. of liver when 12% of casein was fed (Table II, diet I) and 21  $\gamma$ /g. on 24% of casein (Table II, diet II). The total amounts per liver were 61 and 117  $\gamma$ , respectively, on the two diets (Table II). A similar effect of casein on riboflavin was observed under conditions of controlled food intake (Table III). Rats on 12% of casein stored 62  $\gamma$  of the vitamin/liver whereas the average for the group on 24% casein was 99  $\gamma$ . The restriction of food usually caused an increase in the concentration of riboflavin/g. of tissue, although the amount of vitamin/liver decreased somewhat in the restricted animals. This is probably associated with the fact that the livers of partially starved animals decreased in size. Adult animals consuming 12.9 g. of diet III daily contained 17.1  $\gamma$  of riboflavin/g. of liver (Table IV). Those restricted to only 5.5 g. of food contained 24.1  $\gamma$ /g. The total amounts per liver were 155 and 129  $\gamma$ , respectively.

Part of this increase of vitamin/g. of tissue is probably due to the fact that in the restricted animal glycogen and fat are disappearing from the liver at a faster rate than the proteins to which the vitamin is attached. Another factor to be considered is that under conditions of severe restriction animals may lose weight and an increased con-

centration of riboflavin/g. of liver tissue may be partly due to vitamin that has accumulated there from other tissues of the body. However, in most of the present experiments, the restricted animals did not lose weight.

## DISCUSSION

Much evidence is available to indicate that riboflavin retards the rate at which tumors develop when azo dyes are fed to rats (2, 3, 9, 10), whereas none of the other B vitamins possess this property to anything like the same degree (3). Indeed two of them, pyridoxine (3, 16) and biotin (17), actually appear to increase the rate of tumor development under certain experimental conditions. The older evidence on the importance of riboflavin in hepatic tumor formation (1-3) is now strengthened by the observation that the highly carcinogenic azo dyes interfere with the retention of riboflavin in the liver, whereas related non-carcinogenic dyes do not, while the mildly carcinogenic azo dyes are intermediate in their effect upon riboflavin storage (Table IV).

A possible role of riboflavin in tumor formation is further suggested by the fact that liver tumors contain much less riboflavin than normal livers or than relatively normal liver tissue adjacent to a hepatoma. In agreement with others (18-20) we have observed that liver tumors due to *p*-dimethylaminoazobenzene contained 2.1-8.0  $\gamma$  of riboflavin/g. of tissue; as compared to 16-20  $\gamma$ /g. of normal liver from animals on comparable diets. Tumors due to the *m'*-methyl- or *o'*-methyl azo dyes were likewise low in riboflavin. It should be pointed out, however, that the concentration of most other vitamins is also appreciably less in hepatoma tissue than in normal liver (18), which suggests that the results of chemical analyses taken by themselves do not necessarily reflect the true role of a nutrient in carcinogenesis; parallel data on tumor formation must also be considered. In the case of riboflavin, however, both the analytical and biological data lead to the same conclusion, *viz.*, that riboflavin tends to hinder the formation of liver tumors due to azo dyes.

The mechanism by which the carcinogenic azo dyes interfere with the storage of riboflavin in the liver is unknown, although the effect does not appear to be exerted through the medium of the microorganisms of the digestive tract. Schweigert and associates have reported that the feeding of succinyl sulfathiazole to rats fails to affect the con-

centration of riboflavin in the liver and muscle (21), although the drug greatly restricts the activity of the intestinal microorganisms and interferes with the synthesis of certain other vitamins (22, 23).

A more likely possibility is that the carcinogenic azo dye or some derivative disturbs the equilibrium between free and bound riboflavin in the liver. Less riboflavin is retained in the liver on a low protein diet than when the percentage of dietary protein is high (4-6), while several enzymes are known to require specific riboflavin nucleotides as coenzymes. Perhaps the azo dye minimizes the firmness with which riboflavin is anchored to protein in the liver. Should this prove to be the true explanation, it would parallel that offered (24) for the observation that carcinogens like dibenzanthracene interfere with the ability of the liver to retain vitamin A (24, 25).

#### SUMMARY

1. The addition of certain carcinogenic azo dyes to the diet of rats caused a decrease in the riboflavin content of the liver. The decrease appeared to be roughly equivalent to the carcinogenicity of the dye: *m'*-methyl-*p*-dimethylaminoazobenzene was most effective, *p*-dimethylaminoazobenzene and *p*-monomethylaminoazobenzene were fairly effective, whereas *o'*-methyl-*p*-dimethylaminoazobenzene, *p'*-methyl-*p*-dimethylaminoazobenzene, aminoazotoluene, aminoazobenzene or azobenzene had little or no effect.

2. More riboflavin was stored in the liver when the basal diet contained 24% of casein than when 12% was fed. The relative effects of the carcinogens were essentially the same on both diets.

3. The presence of carcinogenic azo dyes in the diet resulted in a decreased food intake, but this was not responsible for the impaired vitamin storage. Rats fed restricted amounts of the control diet free from the dye showed only a slight decrease in the total amount of riboflavin/liver while the concentration of vitamin/g. of liver tissue was usually higher than in rats fed *ad libitum*.

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# On the Excretion of Nicotinic Acid Following a Test Dose of Trigonelline

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## INTRODUCTION

Incidental to other studies on the metabolism of trigonelline (1), the urinary excretion of nicotinic acid was determined by the method of Melnick and Field (2). The conditions of the experiment have already been described (1). It was found that following the injection of a 300 mg. dose of trigonelline sulfate the excretion of nicotinic acid invariably rises, reaching maximum values in 4-12 hours after the test dose was administered. The average apparent maximum rise is 130% above the average basal value, varying between 36.2-300%. An increased excretion of nicotinic acid following injection of trigonelline would indicate that the human body can demethylate trigonelline, with the formation of nicotinic acid and methyl groups.

These results were surprising to us because trigonelline is not known to have any antipellagra activity and cannot substitute for nicotinic acid in preventing the profound growth retardation of rats fed a corn-rich, nicotinic acid-poor diet (3). Furthermore, trigonelline does not possess a lipotropic action (4). On the contrary rats can demethylate N'-methylnicotinamide and this substance has both lipotropic (5) and antipellagra activity (6).

Therefore, we repeated our experiments determining urinary nicotinic acid using the microbiological technic (7), instead of the method of Melnick and Field.

Five normal subjects were kept on a controlled low-niacin diet and, after three 8-hour samples of urine had been collected, 300 mg. of trigonelline were administered orally. The urine was then collected at various intervals of time and analyzed for free and total nicotinic

acid. The latter was determined after a 30 minute acid hydrolysis of the urine. The results are presented in Table I and show that the urinary excretion of nicotinic acid does not increase significantly after the injection of trigonelline.

TABLE I

*Urinary excretion of Nicotinic Acid after Oral Administration of a 300 milligram Test Dose of Trigonelline Sulfate*

All values expressed in micrograms per hour

Hours	PPF		A.S.G.		D.H.N.		T.C.		M.D.L.	
	Free Nicotinic Acid	Total Nicotinic Acid	Free Nicotinic Acid	Total Nicotinic Acid	Free Nicotinic Acid	Total Nicotinic Acid	Free Nicotinic Acid	Total Nicotinic Acid	Free Nicotinic Acid	Total Nicotinic Acid
Before test dose	22.0	39.0	17.5	47.0	23.5	28.0	17.5	23.0	37.5	54.2
	21.6	43.0	10.0	25.0	50.0	85.5	24.7	37.5	25.0	35.0
	25.0	51.0	8.0	27.0	44.0	69.0	36.5	41.0	37.5	44.0
	30.3	—	21.0	37.5	32.5	79.0	23.0	32.0	16.0	26.0
Hourly Average	25.5	44.3	13.0	29.8	42.0	77.7	28.1	36.6	30.5	35.0
After test dose										
4	30.0	30.0	47.0	48.0	47.5	72.2			41.0	75.0
8	12.0	44.0	20.0	40.0	43.0	73.0	16.0	27.5	22.0	35.0
16	32.0	45.0	2.5	33.0	44.0	69.0	23.5	34.0	27.0	40.0
24	10.5	34.0	18.5	41.0	20.5	41.0	29.6	41.0	34.5	41.0
Aver. 1st Day	21.1	39.0	17.5	39.3	36.5	61.5	22.4	34.0	31.0	45.2
40	31.3	52.0	29.5	37.5	38.7	87.5	22.0	28.1	33.0	41.0
48	12.5	43.0	2.5	28.0	15.0	28.0	28.0	40.0	19.6	30.0
Aver. 2nd Day	25.0	49.0	20.5	34.2	31.0	67.5	23.5	32.0	28.5	36.5

We, therefore, feel that the results obtained in our first experiments were probably due to lack of specificity for nicotinic acid of the method of Melnick and Field which probably also determines pyridine derivatives other than nicotinic acid.

## CONCLUSION

The oral administration of trigonelline to 5 human subjects was not followed by a significant increase in the urinary excretion of nicotinic acid.

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# Dietary Deficiencies and Poliomyelitis. Effects of Low Protein and of Low Tryptophan Diets on the Response of Mice to the Lansing Strain of Poliomyelitis Virus \*

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## INTRODUCTION

In 1944 it was reported from this laboratory (1) that a dietary deficiency of either vitamin B<sub>1</sub> or calories increased the resistance of mice to the Lansing strain of poliomyelitis virus. Similar results have been reported subsequently by Rasmussen *et al.* (2). Foster and associates (1) pointed out that their data suggested that the vitamin-deficient animals were more resistant than those on the restricted food intake. In experiments in which the paired feeding technique was used, these same authors (3) not only found that the vitamin deficiency exerted the greater protection, but that the difference between a deficiency of vitamin B<sub>1</sub> and a deficiency of calories on the response of mice to the virus was statistically significant. It was apparent from these results that this effect of the vitamin deficiency could not be entirely due to the resulting anorexia and inanition which are so pronounced in vitamin B<sub>1</sub> deficiency. Since these observations were made, the effects of several other deficiencies have been studied in an attempt to determine whether an insufficient amount of some other

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† With technical assistance of Sachi Takagi.

dietary essentials might produce similar results or whether this phenomenon is more or less specific for a vitamin B<sub>1</sub> deficiency.

All of the work on the chemistry of the viruses has shown that they are composed chiefly of protein. As viruses depend on the host cells for their structural elements it was thought that by limiting the supply of some of these elements which the host itself cannot synthesize, it might be possible to interfere with the activities of the virus. In the present communication experiments are presented which were designed to study the effects of (a) a low protein diet and of (b) a diet deficient in tryptophan on the course of the infection.

### EXPERIMENTAL

Mice at approximately 25 days of age were used as experimental animals and in most experiments they were divided into four groups. Two of the groups were given the deficient diet and the other two the same diet to which had been added a sufficient amount of the factor (protein or tryptophan) under consideration. A few days after the animals had shown little or no gain in weight, one group on each diet was inoculated intracerebrally with a suspension of mouse brain containing between 10 and 50 LD<sub>50</sub> of virus. The other two groups were injected with a suspension of uninfected mouse brain. The animals were weighed daily and, after inoculation, were individually examined 4 times in each 24 hours. This technique enabled the observer to distinguish clearly between the animal dying of poliomyelitis and that dying of dietary deficiency.

*Low Protein Experiments.* Three experiments on the effect of a diet low in protein (casein) were carried out, of which only the third will be presented in detail.

The complete diet (Table I) contained 25% casein, while in the deficient diet the casein content was reduced to 5%, the difference in calories being made up by 20% glucose. The animals were divided into the four groups outlined above; groups 1 and 2 on the complete diet, groups 3 and 4 on the deficient diet. After 10 days of these experimental diets, the mice in groups 1 and 3 were inoculated with virus-containing mouse brain, and groups 2 and 4 with normal mouse brain. The pre- and post-inoculation growth curves are shown in Fig. 1.

Table II presents the result of the inoculation. The cumulative percentages of deaths are given at 2-day intervals for the duration of the experiment of 28 days. With the exception of one animal in group 1 all of the animals which died following inoculation with virus showed signs of poliomyelitis shortly before death. A comparison of the data obtained with groups 1 and 3 shows that the deficiency produced a slight delay in the development of poliomyelitic lesions. The difference

TABLE I  
*Control or Complete Diet*

Diet	Parts	Vitamin B mixture	mg./100 g
Crude casein	25.0	Thiamine chloride	2.0
Cellulose	2.0	Riboflavin	5.0
Salt mixture <sup>1</sup>	4.0	Pyridoxine	0.2
Linseed oil	1.5	Calcium pantothenate	5.0
Wheat germ oil	1.0	Nicotinic acid	10.0
Fish liver oil concentrate	0.008	Inositol	10.0
Glucose <sup>2</sup>	63.5	p-Aminobenzoic acid	10.0
		Choline chloride	30.0
	97.008	Cerelose	3.0
Vitamin B mixture	3.0		

<sup>1</sup> Salts no. 12 (Jones and Foster, '42) (4).

<sup>2</sup> Cerelose, Corn Products Refining Co.

in these two groups was not statistically significant ( $\chi^2$  of 3.8 or more) after the 8th day and, by the 11th day, there was no difference between them. A few days later there had been more deaths in the deficient group than in the control group. This slight difference remained to the end of the experiment, when 70% of the animals on the diet containing

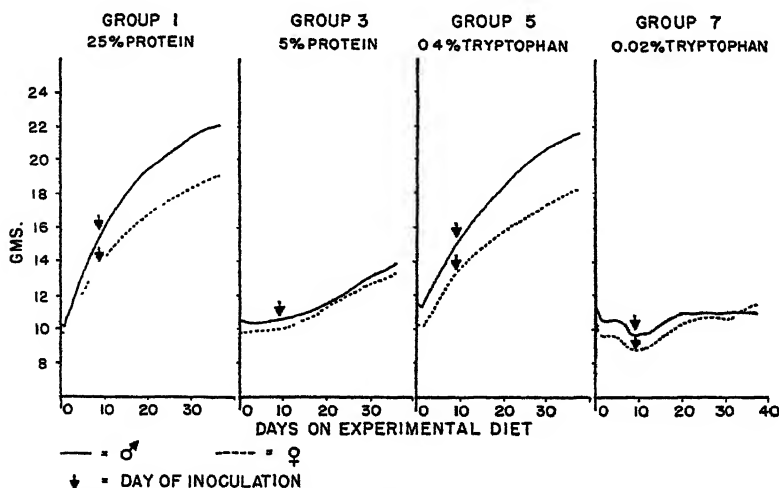


FIG. 1. Effect of Low Protein and of Low Tryptophan on Growth of Mice Inoculated with Poliomyelitis Virus



TABLE II

*Effect of Either Low Protein or Low Tryptophan in the Diet on the Response of Mice to the Virus of Poliomyelitis*

Days after inoculation	Cumulative deaths in per cent of total mice in each group		Days after inoculation	Cumulative deaths in per cent of total mice in each group*	
	Group 1 25% casein	Group 3 5% casein		Group 5 0.4% tryptophan	Group 7 0.02% tryptophan
2	6†	0	2	8‡	0
4	16	4	4	10	0
6	16	4	6	16	2
8	26	12	8	28	6
10	36	31	10	48	10
12	48	45	12	52	18
14	56	57	14	56	38
16	60	69	16	60	48
18	68	73	18	62	56
20	70	76	20	66	60
22	72	78	22	68	62
24	72	80	24	72	66
26	72	82	26	74	68
28	72	84	28	74	70

\* 50, 49, 50 and 48 mice in groups 1, 3, 5, and 7 respectively.

† One animal died in which signs of poliomyelitis were not observed.

‡ Three animals died in which signs of poliomyelitis were not observed.

25% protein had died, compared to 84% on the diet containing 5% protein. This difference, however, is not statistically significant. There were no deaths in either group 2 or 4.

The slight delaying action of the low protein diet was also observed in both the other experiments. In the first experiment, on the 9th day, 18% of the animals in group 1 (high protein diet and injected with virus) were dead as compared with 8% in group 3 (low protein diet and injected with virus). On the 13th day these figures were 54 and 56%, respectively. In the second experiment, the corresponding figures were 28 and 4%, respectively, on the 9th day for groups 1 and 3. In this experiment the delaying action was more pronounced and of longer duration than in either the first or third experiments. The difference in deaths in these two groups of animals was statistically significant to the 14th day, and it was not until the 22nd day that they became nearly equal at 86 and 82%.

*Low Tryptophan Experiments.* It is possible that at the level of 5% casein the amino acids which are the limiting factors for growth of animals may not be the ones which are the limiting factors for the needs of the virus. However, it is conceivable that there are amino acids, essential to the needs of the virus, that are quantitatively different from those essential to the needs of the host. Even in a 5% casein basal diet there may be enough of those amino acids to support the virus, whereas another source of protein may result in an amino acid deficiency for the virus but not for the host. Whether or not this is true can only be determined by a study of individual amino acids.

Two experiments were conducted in which the effects of a deficiency of tryptophan on the response of mice to the virus of poliomyelitis were studied, but the details of only the second test will be presented here. The basal low-tryptophan diet used was a modification of the Osborne and Mendel zein diet (5). The general composition of the diet was the same as that given in Table I, except that the protein was furnished by 25% zein and the following amino acids were added: *dl*-lysine, 0.4%; *l*(-)-cystine, 0.3%; and *dl*-methionine, 0.3%. Glucose was supplied to make 100%. This diet produced good growth in mice when 0.4% of *dl*-tryptophan was added, and there was an immediate loss in weight when the tryptophan was omitted (Fig. 1). Tryptophan at a level of 0.02% was included in the deficient diet to prevent an undue number of the animals from dying of the deficiency.

There were 4 groups of animals as previously described. Groups 5 and 6 were given the complete diet (0.4% tryptophan). Groups 7 and 8 were given the deficient diet (0.02% tryptophan). After 8 days on the experimental diets, groups 5 and 7 were inoculated with the virus and groups 6 and 8 were injected with uninfected brain. At this time the animals were losing weight rather rapidly, and the amount of tryptophan in the diet of groups 7 and 8 was increased to 0.03%. Eleven days later this was again reduced to 0.02%.

The effect of the tryptophan deficiency on the response of the animals to the virus was much the same as that of the deficiency of protein (Table II). However, the delaying action of the tryptophan deficiency for the first two weeks following inoculation was considerably more pronounced than that of the protein deficiency. By the 10th day, 48% of the animals on the complete diet had died as compared with only 10% on the deficient diet. The difference between the two groups was statistically significant from about the 6th to the 18th day. In fact,

during this period the protection was nearly as great as that obtained in some of the earlier experiments from a deficiency of vitamin B<sub>1</sub>, but, unlike vitamin B<sub>1</sub>, the effect of the deficiency of tryptophan was of short duration and appeared only to delay the action of the virus and not to prevent it. Of the animals on the complete diet which succumbed, 3 died before any signs of poliomyelitis were seen. Paralysis was observed in all of the animals that died in group 7 (deficient and inoculated with virus). Five of the animals in group 8 (deficient and injected with uninfected brain) died before the experiment was terminated. This would indicate that the animals of both groups on the diet low in tryptophan were in a rather advanced state of deficiency. In spite of this marked deficiency, the low-tryptophan intake exerted but a temporary resistance against the virus. The first tryptophan experiment gave results which agreed with those just described.

The second experiment of the low protein studies was conducted simultaneously with the first tryptophan experiment and with animals taken from the same litters. This was also true for the third protein experiment and the second tryptophan experiment. This allowed a direct comparison between the complete casein diet and the complete zein diet. In the first experiment in which this comparison was made, 60% of the animals on the zein diet died in contrast to 88% on the high casein diet. In the next experiment (third protein and second tryptophane experiments), however, there was no difference between these two groups. In this particular case the rather marked variation in the proportion of the various amino acids in these two proteins had little or no effect on the activity of the virus.

In connection with the first of these two experiments just discussed, we also had occasion to repeat the experiment on the effect of a vitamin B<sub>1</sub> deficiency. The results were in complete agreement with the observations already reported.

#### ACKNOWLEDGMENT

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#### SUMMARY

In three experiments the effects of a diet low in protein on the response of mice to the virus of poliomyelitis have been studied.

Except for a delaying action as to the onset of symptoms for the first 10-14 days following the inoculation, the protein deficiency had no effect on the resistance of mice to this virus.

Two similar experiments were conducted on the effects of a deficiency of tryptophan. Here again, on the deficient diet there was delayed appearance of poliomyelitis symptoms which was more pronounced than in the deficiency of protein. However, at the termination of the experiments (28 days after inoculation) the incidence of poliomyelitis and the number of deaths were approximately the same on the deficient and complete diets.

Except for a delaying action during the first two weeks following inoculation, a marked deficiency of either protein or tryptophan neither increased nor decreased the susceptibility of mice to the virus of poliomyelitis.

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# Isolation and Identification of Fatty Acids as Bis-(*p*-dimethylaminophenyl)-ureides

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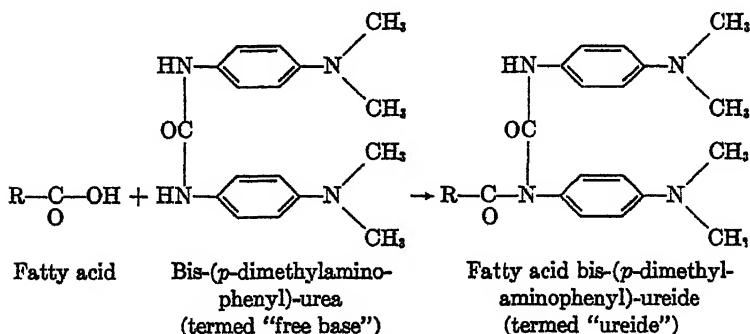
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## INTRODUCTION

For biochemical purposes we need methods which permit simple isolation and identification of small amounts of free fatty acids even in the presence of large amounts of neutral fats.

Of the hitherto known methods, microdistillation is difficult and cannot be applied to hydroxy fatty acids. Separation by means of salts leads to losses and, for the preparation of crystalline nitrobenzyl esters, the isolation of fatty acids and the preparation of dry sodium salts is necessary.

A good procedure, which avoids these disadvantages, is the isolation of free fatty acids as crystalline ureides of bis-(*p*-dimethylamino-phenyl)-urea. These ureides are formed directly by merely boiling a solution of the fatty acid with a solution of the urea derivative in ether. As, in contradistinction to fatty acids and the urea compound, most of the ureides are only slightly soluble in ether, the ureide crystallizes directly from the ether, even in the presence of large amounts of neutral fat.



The ready formation of ureides was discovered by C. Schall, 1901, who used diphenylurea. Since the ureides thus formed are not very stable, F. Zetsche *et al* (1938 I and II) investigated other substituted diphenylureas and found the above mentioned free base especially adaptable.

In addition to ureides of aromatic acids (V) these authors also synthesized ureides of some fatty acids (III and II). They found that  $\alpha, \beta$ -unsaturated acids yielded yellow ureides. R. Tulus (1944) synthesized ureides of higher homologous fatty acids, also  $\alpha, \beta$ -unsaturated, and confirmed the results of Zetsche and his collaborators.

We prepared ureides of the normal fatty acids from  $C_1$  to  $C_{20}$  (except  $C_{13}$ ,  $C_{15}$ ,  $C_{17}$  and  $C_{19}$ ). We also prepared ureides of  $\alpha, \beta$ -unsaturated acids, of some higher  $\beta$ -hydroxy fatty acids, and of naturally occurring unsaturated fatty acids. Their melting points, behavior and solubilities in ether, acetone, ethanol and ethyl acetate have also been investigated. To investigate the possibility of separation of homologous ureides by crystallization, some solubility-temperature curves have also been drawn.

## EXPERIMENTAL

The bis-(*p*-dimethylaminophenyl)-urea was prepared *via* dimethyl-aniline  $\rightarrow$  *p*-nitrosodimethylaniline  $\rightarrow$  *p*-aminodimethylaniline  $\rightarrow$  +  $CS_2 \rightarrow$  bis-(*p*-dimethylaminophenyl)-thiourea  $\rightarrow$  free base (Zetsche, Röttger; II). The free base, white crystals, m. p. 86–89°C., is soluble at 21°C. in ether 6.5%, in acetone 14.2%, in alcohol 0.6%, and in ethyl acetate (acid-free) 12.1%. The free base is insoluble in water but readily soluble in acids.

For the preparation of the ureides a 1–4% solution of the fatty acid in ether with approximately the necessary molar amount of a saturated clear solution of the base in ether is boiled under reflux for 2–3 hours. The ureides crystallize from the liquid chiefly during boiling, but sometimes after cooling in an icebox or after evaporation of part of the ether. Only the monoureides, containing one mole fatty acid and one mole base form with fatty acids, regardless of an excess of one or the other.

The formation of the ureide is not quantitative; the yield is about 80% under our conditions. The yield is nearly the same from dry or wet ether, but presence of free water decreases the yield. The ureides, even of high molecular weight fatty acids such as stearic acid, are readily soluble in 5% HCl (but not in 0.1%), although they are quite insoluble in water and have practically no basic properties. Attempts to titrate them in alcohol-benzene solution with HCl failed. Nevertheless, it is possible to extract the ureides quantitatively from ethereal solutions by means of 5% HCl. The crystalline ureide can be regained unchanged from this solution by precipitation with cold alkali.

TABLE I

*Bis-(p-dimethylaminophenyl)-ureides of Saturated Fatty Acids*

Name of Acid	C Number	M. pt. °C.	Per cent Soluble at 20°C.				Per cent Nitrogen	
			Ether	Ethanol	Acetone	Ethyl Acetate	Calculated	Found
Formic	C <sub>1</sub>	152 (154, Z)	0.18	0.22	0.86	0.58	—	—
Acetic	C <sub>2</sub>	152 (149, Z)	0.13	0.11	0.92	0.49	—	—
Propionic	C <sub>3</sub>	161 (120, Z)	0.035	0.032	0.23	0.16	—	—
Butyric	C <sub>4</sub>	153	0.08	0.12	0.53	0.47	15.22	15.47
n-Valeric	C <sub>5</sub>	150.5	0.20	0.10	1.07	1.05	14.66	14.76
Caproic	C <sub>6</sub>	143	0.18	0.10	0.82	0.89	14.14	14.33
Heptylic	C <sub>7</sub>	134	0.20	0.09	1.19	1.01	13.66	13.45
Caprylic	C <sub>8</sub>	136.5	0.32	0.15	1.18	1.14	13.21	13.86
Pelargonic	C <sub>9</sub>	126	0.56	0.34	2.97	4.34	12.78	13.24
Capric	C <sub>10</sub>	141.5	0.09	0.035	0.30	0.33	12.39	12.44
Undecylic	C <sub>11</sub>	140	0.053	0.032	0.16	0.16	12.01	12.09
Lauric	C <sub>12</sub>	133	0.66	0.13	1.10	1.54	11.66	11.79
Myristic	C <sub>14</sub>	128	0.42	0.14	0.78	1.47	11.02	11.41
Palmitic	C <sub>16</sub>	129.5 (120, Z)	0.77	0.05	0.45	0.65	—	—
Stearic	C <sub>18</sub>	122	0.32	0.12	0.61	1.2	9.93	10.08
Arachidic	C <sub>20</sub>	117	0.13	0.03	0.075	0.178	9.46	9.64



TABLE I (Continued)  
*Ureides of  $\alpha$ ,  $\beta$ -Unsaturated Fatty Acids*

Name of Acid	C Number	M. pt. °C.	Per cent Soluble at 20°C.				Per cent Nitrogen	
			Ether	Ethanol	Acetone	Ethyl Acetate	Calculated	Found
Crotonic	C <sub>4</sub>	148 (150, T)	0.11	0.08	1.02	0.43	—	—
$\beta$ -Methylcrotonic	C <sub>5</sub>	141	0.30	0.13	2.11	1.04	14.80	15.10
1-Pentenoic	C <sub>5</sub>	140	0.78	0.56	3.68	3.35	14.80	15.10
1-Nonenoic	C <sub>9</sub>	121 (121, T)	0.74	0.15	3.29	1.81	—	—
1-Decenoic	C <sub>10</sub>	128.5 (131, T)	0.93	0.19	2.40	2.41	—	—
1-Hendecenoic	C <sub>11</sub>	127.5 (128, T)	0.38	0.10	1.44	0.97	—	—
1-Dodecenoic	C <sub>12</sub>	127 (126, T)	1.548	0.22	7.18	5.02		
1-Tridecenoic	C <sub>13</sub>	123 (121, T)	5.09	0.52	9.04	—	—	—
1-Tetradecenoic	C <sub>14</sub>	111 (111, T)	9.59	1.17	12.83	—	—	—

*Ureides of other Unsaturated Fatty Acids*

10-Hendecylenic	C <sub>11</sub>	136	0.065	0.193	0.575	1.15	12.07	12.23
Oleic	C <sub>18</sub>	103 (101, Z)	3.24	0.17	3.98	4.56	—	—
Stearolic	C <sub>18</sub>	114	1.47	0.09	2.5	2.78	10.	10.01
Ricinoleic	C <sub>18</sub>	122	0.15	0.50	0.54	2.16	9.89	10.63
9,11-Linoleic	C <sub>18</sub>	107	1.37	0.08	7.42	2.83	10.	10.08
9,12-Linoleic	C <sub>18</sub>	91	3.58	0.15	6.0	7.2	10.	10.42
9,12,15-Linolenic	C <sub>18</sub>	91.5	2.59	0.15	7.03	8.24	10.04	10.55

TABLE I (Continued)  
*Ureides of  $\beta$ -Hydroxy Fatty Acids (Racemic Mixture of d- and l-Acid)*

Name of Acid	C Number	M <sub>C</sub> <sup>pt</sup>	Per cent Soluble at 20°C.				Per cent Nitrogen	
			Ether	Ethanol	Acetone	Ethyl Acetate	Calculated	Found
$\beta$ -Hydroxypelargonic	C <sub>9</sub>	134 (Dec.)	1.12	0.95	2.72	—	12.33	12.20
$\beta$ -Hydroxycapric	C <sub>10</sub>	131 (Dec.)	0.68	1.59	4.10	—	11.97	12.17
$\beta$ -Hydroxyhendecylic	C <sub>11</sub>	120	0.25	0.61	1.07	1.27	11.61	11.40
$\beta$ -Hydroxylauric	C <sub>12</sub>	118	1.12	3.34	4.27	5.82	11.29	11.16
$\beta$ -Hydroxytridecanoic	C <sub>13</sub>	116.5	3.41	5.77	12.92	9.61	10.98	10.69

In this way free fatty acids can be separated easily from neutral fats. As, contrary to the alkaline extraction of free fatty acids previously used, no emulsion forms between the ether and water layers with this kind of extraction, the method is superior to the ones formerly

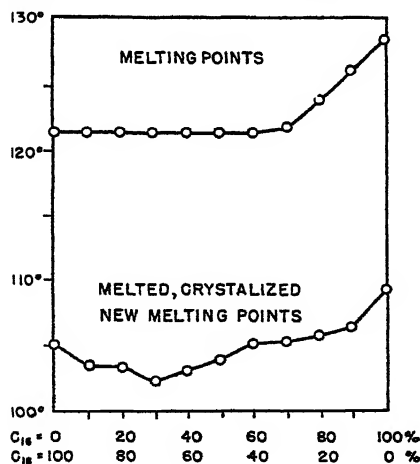


FIG. 1

Melting Point Depression Curve

Upper curve—original m. pt.

Lower curve—after crystallizing and remelting.

used. In addition, the free fatty acids are isolated in crystalline form, in contrast to the undefined soaps obtained in alkaline extraction.

As the temperature-solubility curves are very steep, even such small amounts as a few mg. of the ureides can be recrystallized and purified 3-4 times without great losses.

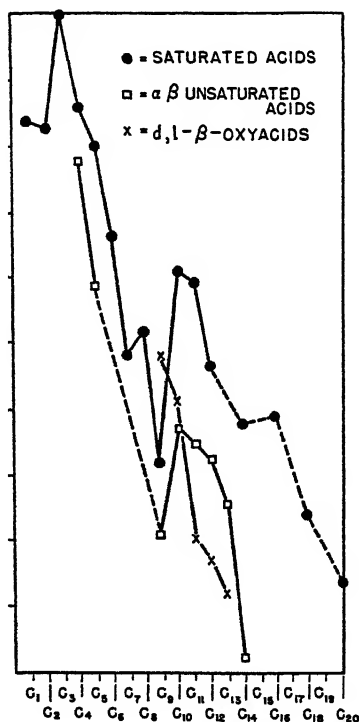


FIG. 2

#### Melting Points of Ureides of Different Fatty Acids

Recovery of the free acids is brought about by heating the ureides with 5% alcoholic KOH, as mentioned by Zetsche (II).

Some disadvantages of the ureides are mentioned by Zetsche. One is the instability of the ureides at temperatures above 150°, although this does not interfere with recrystallization from low-boiling solvents. With ethyl acetate, however, the mother liquor sometimes becomes brown, especially with hydroxy acids, although the crystals separating from ethyl acetate are white and have the proper melting point.

Another disadvantage is that the mutual depression of melting points between two homologous ureides is weak. A depression curve has been drawn. Furthermore, the melting points have an interval of about 1°C. Melted ureides recrystallize when cooled, their melting point then being about 15° lower, but this rises to the old point after one recrystallization from a solvent.

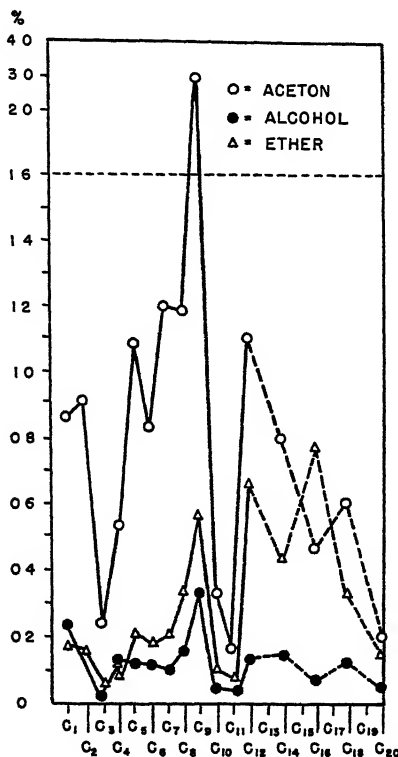


FIG. 3

Solubilities of Ureides of Saturated Fatty Acids in Acetone, Alcohol, Ether

Not only the melting point curves, but also the solubility curves show regularities quite different from the usual curves for properties of free fatty acids. Often there are very great differences of solubilities between two neighboring homologous ureides; differences which can easily be used for separative purposes.

It is to be noted that all acids with 11 C atoms (also with 3 C atoms) show a minimum solubility independent of chemical structure; regard-

less of whether they are saturated, unsaturated or  $\beta$ -hydroxy acids. To obtain a better picture, all data are presented in the form of diagrams following the Table.

In the Table, names, C-number, melting point, N-content calculated and found by Dumas analyses, and solubilities of the different fatty acids are given.

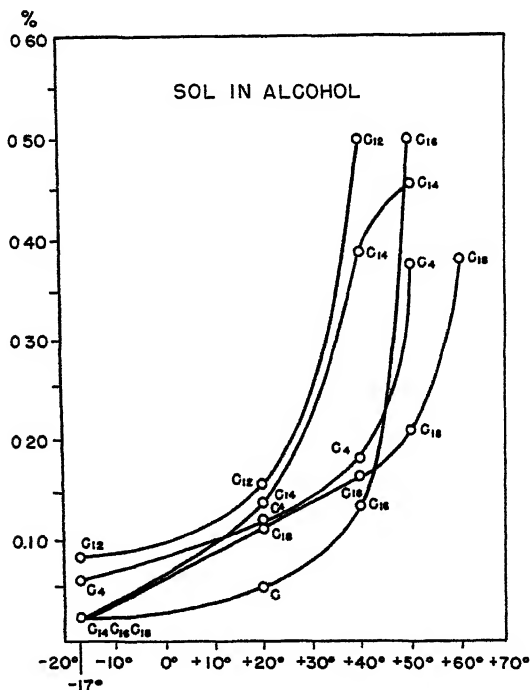


FIG. 4

Dependence of Solubility of Ureides of Some Saturated Fatty Acids on Temperature In Alcohol

To the melting points of ureides newly prepared, but previously synthesized by Zetsche (Z) or Tulus (T), the old melting points are added in parentheses.

#### *Mutual Melting Point Depression Curve for Ureides*

Accurately weighed samples of the ureides of stearic (m.p. 122°C.) and palmitic acids (m.p. 129.5°C.) were intimately mixed on a watch

glass. To homogenize, 3 ml. of ether were added and evaporated at room temperature. From these mixtures the melting points were determined in a  $\text{H}_2\text{SO}_4$ -bath with mechanical stirring using a thermometer graduated in  $0.2^\circ\text{C}$ .

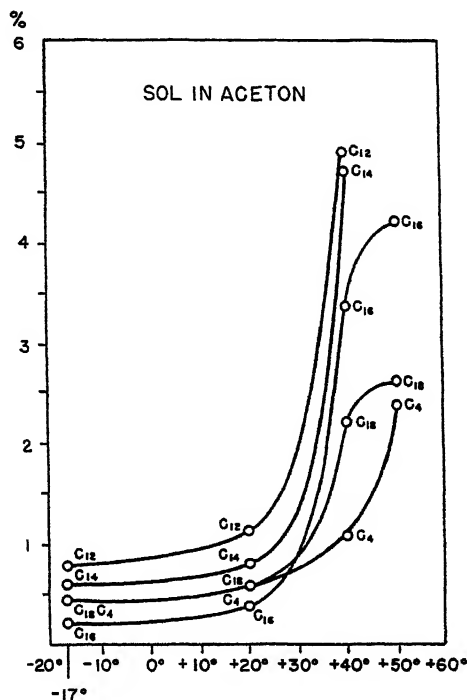


FIG. 5

Dependence of Solubility of Ureides of Some Saturated Fatty Acids on Temperature In Acetone

#### *Direct Separation of Fatty Acids from Neutral Lipides*

Three hundred mg. of propionic acid plus 5.0 g. of pure triolein plus 1 g. of free base in 40 ml. of ether were heated for 2 hrs. under a reflux. Soon crystals appeared. After 2 hours cooling in an icebox 1.11 g. of crystalline propionic acid ureide (m.p.  $155\text{--}158^\circ\text{C}$ .) could be isolated by filtration. After one recrystallization from acetone the m.p. was  $161^\circ\text{C}$ . All other fatty acids can be separated in the same way. Excess free base can be readily eliminated from the ethereal solution by

merely shaking with dilute HCl, so that a practically pure solution of unaltered triglyceride in ether remains.

### *Separation of Ureides from Lipides*

As traces of a formed ureide, unprecipitated because of its solubility in ether (as olein ureide), may remain in a lipid solution, the following method was employed to remove practically all ureide.

Two hundred and fifty mg. of pure stearic acid ureide (m.p. 122°C.) were dissolved in 100 ml. of pure ether and then extracted 4 times with 50 ml. portions of 5% HCl. After separation the evaporated ether contained only 5 mg. of crystals, thus showing that 98% of the ureide had been removed. The stearic acid ureide can be precipitated unaltered from the clear solution in HCl by addition of alkali (m.p. 121°C.).

### SUMMARY

The direct isolation of free fatty acids from fat mixtures takes place readily by the mere heating of their ethereal solution with bis-(*p*-dimethylaminophenyl)-urea. Crystalline fatty acid ureides precipitate.

Properties, melting points, solubilities in ether, acetone, ethanol and ethyl acetate, of the ureides of saturated fatty acids from C<sub>1</sub> to C<sub>20</sub>, of some unsaturated fatty acids and of  $\beta$ -hydroxy fatty acids are described.

The introduction of this procedure in the analytical chemistry of fats is recommended.

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# The Metabolism of $\alpha$ -, $\beta$ -, $\gamma$ -, and $\delta$ -Keto Fatty Acids in Minced Tissue of Warm-Blooded Animals

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## INTRODUCTION

Pyruvic and acetoacetic acid are partly reduced in minced tissue to the corresponding hydroxy acids, lactic acid and  $\beta$ -hydroxybutyric acid, and partly metabolized otherwise.

Other  $\alpha$ - and  $\beta$ -keto acids, for instance,  $\alpha$ -ketoglutaric acid and oxaloacetic acid, react in an analogous manner.

The metabolism of higher  $\alpha$ - and  $\beta$ -keto acids is almost unknown.  $\alpha$ -Ketobutyric acid is metabolized by the brain (Long, 1943). Higher  $\beta$ -keto fatty acids, as intermediate products of Knoop's  $\beta$ -oxidation, are probably metabolized *via* the formation of citric acid (Breusch, 1943). On the other hand, higher  $\beta$ -hydroxy fatty acids, up to  $C_{14}$ , are hydrogen donors in cell oxidations (Lang, 1939; Breusch, Tulus, 1943).

The behavior of  $\gamma$ - and  $\delta$ -keto fatty acids is practically uninvestigated. Only Thierfelder and Schempp (1921) showed, that  $\gamma$ -keto- $\gamma$ -phenylbutyric acid, given orally to a dog, was excreted in the urine as  $\gamma$ -hydroxy- $\gamma$ -phenylbutyric acid to the extent of about 50%. But the reduction in this case is no proof of the general behavior of  $\gamma$ -keto fatty acids, as in this acid the keto group can enolize under the influence of the benzene double bond, while  $\gamma$ -ketovaleric acid (levulinic acid), is not enolized even in traces.

Herrmann and Zentner (1938), feeding levulinic acid to a dog, found an acidification of the urine. They did not isolate the levulinic acid or any other product from the urine.

In the present publication it is shown, that  $\alpha$ -ketopropionic acid,  $\beta$ -ketobutyric acid and  $\beta$ -ketocaprylic acid are reduced and, respectively, metabolized by minced tissue of warm-blooded animals (used immediately *post mortem*), while  $\gamma$ -ketovaleric acid (levulinic acid) and  $\delta$ -ketocaproic acid remain unchanged under these conditions.

Thus it seems, that only  $\alpha$ - and  $\beta$ -keto groups of fatty acids can be reduced or attacked by the tissue enzymes of warm-blooded animals, while  $\gamma$ - and  $\delta$ -keto groups cannot.



This agrees with the investigations of G. Fischer (1940), who found that double bonds in the  $\alpha$ ,  $\beta$ -position to alcohol, aldehyde and carboxyl groups are hydrogenated by yeast, but not  $\beta$ ,  $\gamma$  or  $\gamma$ ,  $\delta$  double bonds. The transformation stearic acid  $\rightleftharpoons$  oleic acid depends on another enzymatic mechanism.

Only the substituents attached to the  $\alpha$ - and  $\beta$ -C atoms of acids, aldehydes and alcohols seem to be sufficiently mobile to be metabolized by cell enzymes. This also clarifies the special position and extreme reducibility of oxaloacetic acid by tissues, which is simultaneously an  $\alpha$ - and  $\beta$ -keto acid.

It has been shown in earlier publications on the reduction of oxaloacetic acid (Breusch, 1942) and the breakdown of citric acid (Breusch and Tulus, 1946), that there exist two groups of tissues, one capable and one incapable of metabolizing the above-mentioned substances.

In the case of  $\alpha$ - and  $\beta$ -keto acids one group of tissues, liver, muscle, kidney, pancreas and, to a smaller extent, brain, is able to metabolize, while the second group, lung, spleen, placenta and embryonic muscle, is unable to do so (within the limits of error of our analytical methods).

## EXPERIMENTAL

### *Preparation of the Keto Acids*

$\beta$ -Ketocaprylic acid was prepared according to Bouveault and Bongert (1902). The acid was colorless, crystalline, and had a melting point of 71–72°C. with  $\text{CO}_2$ -evolution.  $\delta$ -Ketocaproic acid was synthesized by condensation of  $\beta$ -chloropropionic acid ethyl ester with acetoacetic acid ethyl ester, and saponification of the  $\alpha$ -acetylglutaric acid diethyl ester formed according to Bentley and Perkin (1896). The acid used had a melting point of 34°C. (as hydrate). Pyruvic acid,  $\beta$ -ketobutyric acid and levulinic acid had been freshly synthesized in our laboratory and were of high purity.

### *Methods*

The animals used, cats and pigeons, were killed by a blow on the head, the organs immediately removed and minced in a Latapie mincer. Fifteen g. of minced tissue were incubated in 30 ml. of Ringer-phosphate solution (4 parts of Ringer without  $\text{Ca}^{++}$  and 1 part *m*/10 phosphate buffer pH 7.4), containing 5–20 mg. of the neutralized keto acid. Shaking took place in 250 ml. Erlenmeyer flasks, aerobically at 38°C. Immediately after 15 and after 30 minutes, 5 ml. aliquots of the homogeneous suspension were pipetted out, deproteinized, and the amount of keto acid analyzed, as shown below. The limits of error of the different methods are about 10%. The deviations in all values, reckoned as positively metabolized, are 40–100% of the keto acid present at the beginning of the experiment.

*Analytical*

For *pyruvic acid* the method of Szent-Györgyi, Straub and Bruckner (1936) was adopted. *Lactic acid* was analyzed according to Friedemann, Cotonio and Shaffer (1927). For the estimation of  $\beta$ -ketobutyric acid 8 ml. of the tissue suspension were deproteinized with 4 ml. of 20%  $\text{Na}_2\text{WO}_4$  solution and 4 ml. of 15%  $\text{H}_2\text{SO}_4$ . Ten ml. of the filtrate plus 5 ml. of  $\text{H}_2\text{O}$  were distilled in an all glass apparatus. The distillate, 10 ml., and 10 ml. of 60% KOH solution, and 1 ml. of a 10% solution of salicylaldehyde in alcohol, were heated for 20 min. to  $45^\circ\text{C}$ . After cooling the red color was measured in a Zeiss Stufenphotometer, using filter S 53 and a 10 mm. cuvette. Free  $\beta$ -ketocaprylic acid ( $\text{C}_8$ ) is only slightly soluble in water. Therefore the following procedure was adopted for estimation: 5 ml. of the tissue-keto acid suspension were put in the distillation vessel of an all glass distillation apparatus, together with 60 ml. of a 10%  $\text{Na}_2\text{SO}_4$ -5%  $\text{H}_2\text{SO}_4$  solution. From this 40 ml., containing practically all the obtainable methylamylketone formed from  $\beta$ -ketocaprylic acid by boiling with acids, were distilled directly into the distillation vessel of a second all glass distillation apparatus. From this second vessel 4 ml., now containing all the methylamylketone, were distilled into a graduated test tube. Two-tenths ml. ethanol, 0.3 ml. salicylaldehyde and 2 ml. concentrated  $\text{H}_2\text{SO}_4$  were added. The whole was vigorously shaken, heated for 15 minutes in a boiling water-bath and cooled. Then 5 ml. ethanol were added and the red color compared with a blank in a Zeiss Stufenphotometer, using filter S 53 and a 5 mm. cuvette. This method, like the following ones, is a variation of the method elaborated for the estimation of higher methyl ketones by Thaler and Geist (1939). Because of the lipophilic character of both the acid and the ketone, 30-40% of the theoretical quantity remains absorbed in the tissues, as compared with solutions of the free acid. The same happens with tissue suspensions which have been boiled before adding the keto acid. For different tissues, according to their varying contents of lipides, the amount adsorbed varies. The percentage error remains the same within 5% in repeated estimations, when a suspension is allowed to stand for some hours to inactivate the redox enzymes before adding the keto acid. A mere decarboxylation of the keto acid while being incubated, is not measured by this method. For the estimation of *levulinic acid* ( $\gamma$ -ketovaleric acid) 8 ml. of the tissue suspension were pipetted into 4 ml. of a 25%  $\text{Na}_2\text{WO}_4$  solution and than 4 ml. of 15%  $\text{H}_2\text{SO}_4$  added. From the filtrate 4 ml. were incubated with 0.2 ml. ethanol, 0.3 ml. salicylaldehyde and 2 ml. of concentrated  $\text{H}_2\text{SO}_4$ , the mixture vigorously shaken and heated for 15 minutes in a boiling water-bath. After cooling the mixture, 5 ml. ethanol were added. The red color was measured in a Stufenphotometer as before. Two-tenths mg. levulinic acid gave an extinction coefficient of 0.50; 0.4 mg. gave 0.92; 0.6 mg. gave 1.26.

For the estimation of  $\delta$ -ketocaproic acid 5 ml. of the tissue-acid suspension were pipetted into 2 ml. of a 25% trichloroacetic acid solution. After 3 hours standing and filtration, 2.5 ml. of the clear filtrate plus 1.5 ml. water were treated with salicylaldehyde and  $\text{H}_2\text{SO}_4$  and colorimetrically measured as above. Two-tenths mg. of  $\delta$ -ketocaproic acid gave an extinction coefficient of 0.35; 0.4 mg. gave 0.74; 0.6 mg. gave 1.11.

## RESULTS

In the following table mg. of metabolized keto acids/100 g. wet tissue/hr. are given, calculated from 15 g. tissue and the quantities metabolized in the first 15 minutes. Changes under 10% of the acids added are reckoned as 0, because they are within the limits of error. All values are averages of several repetitions, usually not differing by more than 10% from each other. All values are corrected, if necessary. Blank values, for example, for lactic acid, are subtracted.

For comparison, the values of the capacity of different tissues to reduce oxaloacetic acid, are added in the last column. The values are taken from Breusch and Tulus (1946).

Tissue	Metabolized keto acid mg./100 g. wet tissue/hour						Oxaloacetic acid disappeared
	$\alpha$ -Keto-propionic acid disapp.	Lactic acid formed	$\beta$ -Keto-butyric acid disapp.	$\beta$ -Keto-caprylic acid (Cs) disapp.	$\gamma$ -Keto-valeric acid disapp.	$\delta$ -Keto-caproic acid disapp.	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Liver, cat	420	250	800	25	0	0	1500
Kidney, cat	210	90	560	5	0	0	2000
Heart muscle, cat	—	—	210	—	0	0	—
Muscle, pigeon	120	100	180	10	0	0	2400
Brain, cat	60	40	160	0	0	0	200
Pancreas, cat	30	30	—	0	—	0	600
Lung, cat	0	0	0	0	0	0	0
Spleen, cat	0	0	0	0	0	0	0
Placenta, cat	0	0	0	0	0	0	0
Embryonic muscle, cat	30	20	0	0	0	0	0
Parotid, cat	0	—	0	—	—	0	0

## SUMMARY

The capacity of minced tissue, immediately after death, to metabolize  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -keto acids, has been investigated.

$\alpha$ -Keto acid (pyruvic acid) and  $\beta$ -keto acids ( $\beta$ -ketobutyric acid and  $\beta$ -ketocaprylic acid) disappear.  $\gamma$ -Ketovaleric acid (levulinic acid) and  $\delta$ -ketocaproic acid are not metabolized by any minced tissue.

As shown in the reduction of oxaloacetic acid and the breakdown of citric acid there exist two groups of tissues; one, liver, kidney, muscle,

brain and pancreas, metabolizing  $\alpha$ - and  $\beta$ -keto acids, and a second group, lung, spleen, placenta and embryonic muscle, unable to do so.

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# A New Rapid Method for the Determination of Serum Albumin and Globulin by Ultraviolet Absorption

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## INTRODUCTION

Many methods have been developed for determining the total protein concentration of serum. These methods are usually based upon one of the following: specific gravity, colorimetric determinations for tyrosine or peptide linkages, nitrogen content, turbidimetric procedures, viscosity, gravimetric procedures, refractive index and electrophoretic mobility. The most commonly used procedures involve the determination of the serum specific gravity because the techniques are simple and rapid and the results are reproducible.

In contrast to the many methods used to determine the total protein concentration only three procedures which are basically different have been used to any extent to determine serum albumin and globulin.<sup>1</sup> These methods make use of the differences in solubility, electrophoretic mobility or viscosity between albumin and globulin. Usually the albumin and globulin are separated by precipitating the globulin with ammonium or sodium sulfate and then determining the protein concentration in the original sample and the filtrate by one of the methods given above.

Outside of making use of the differences in solubility, electrophoretic mobility and viscosity of albumin and globulin in order to determine these proteins, no use has been made of other characteristics that distinguish albumin from globulin, *e.g.*, molecular weight, osmotic activity, various amino acid contents, *etc.* The present investigation is concerned with the use of ultraviolet absorption spectroscopy to determine these proteins.

In 1928 Campbell Smith (1) showed that a protein-free filtrate of serum gave an ultraviolet absorption curve very similar to that of a simple solution of uric acid. The concentration of uric acid required to give this absorption was approximately that present in plasma. He suggested (2) that it may be possible to determine the

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<sup>1</sup> For this study the term serum globulin will be taken to include all of the various globulins of serum.

serum and cerebrospinal fluid albumin-globulin ratio spectroscopically. However, no further studies were reported. It is likely that the method was not successful because the values of  $E_{1\text{cm}}^{1\%}$  of albumin and globulin were not correct.

The results of some recent studies on plasma proteins (3) indicated that it may be possible to determine serum albumin and globulin very easily and rapidly by using the ultraviolet absorption techniques. Absorption curves for fibrinogen, purified albumin and  $\gamma$ -globulin at pH 2 are given in Fig. 1. The ultraviolet absorption curves of normal serum have the same form as those of the purified proteins.

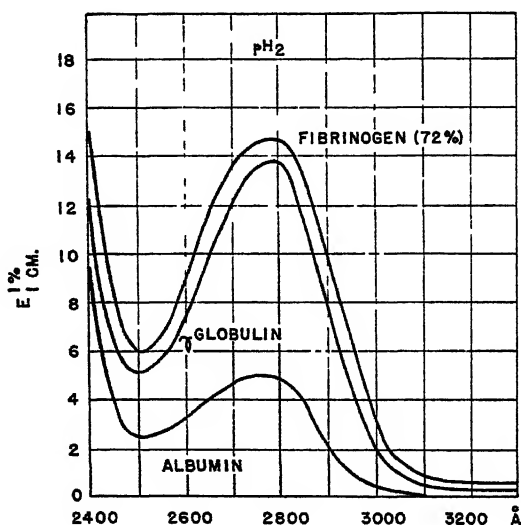


FIG. 1

## METHODS AND RESULTS

To standardize the method, 20 normal sera were studied. These sera were obtained from ten senior medical students and ten conscientious objectors. It was decided to study all of the serum samples at the arbitrarily chosen pH of 2. Most absorption readings were made at 2775 Å because, as will be evident later (Table I), serum has a maximum absorption at that wave length.

It was first necessary to determine the substances responsible for the ultraviolet absorption of serum. This was done by dissolving the

TABLE I  
*Extinction Coefficient of Serum*

No.	2760 Å	2775 Å	2790 Å
1	49.0	49.1	48.8
2	52.5	52.9	52.5
3	48.8	49.1	48.8
4	51.8	52.1	51.9
5	55.4	55.8	55.1
6	54.8	55.1	54.8
7	50.1	50.5	50.1
8	54.0	54.3	54.0
9	53.0	53.3	53.3

various organic substances of serum in 0.01 *N* HCl whenever possible. The results are shown in Table II.

However, there are many other substances in serum. While they do not have any appreciable ultraviolet absorption when present in their normal concentrations, they may be markedly elevated in certain pathological conditions and give moderate absorption.

It was found that the serum should be diluted 1:100 to obtain good density values with cuvettes 1.00 cm. in thickness. If the serum

TABLE II

Substance	Conc. mg-%, pH 2.0	Ext. coef 2775 Å
Globulin	2.3 (g.)	34.5
Albumin	5.0 (g.)	25.0
Uric acid	4.4	2.92
Bilirubin*	0.5	0.14
Cholesterol†	200	0.12
Urea (as nitrogen)	15	0
Creatine	7	0
Creatinine	2	0
Glucose	100	0
Lactic acid	20	0
$\beta$ -Hydroxybutyric acid	1	0
Acetoacetic acid	1	0
Acetone	1	0
Sodium sulfamerazine	10	2.73
Sodium sulfadiazine	10	3.35
Sodium sulfathiazole	10	4.95
Penicillin	100 units/cc.	0

\* The bilirubin solution had a pH of 13.

† The cholesterol was dissolved in chloroform.



were diluted with 0.01 N HCl so that the final solution had a pH of 2, the maximum absorption was always at 2775 Å. If one assumes that all of the various serum globulins have the same  $E_{1\text{ cm}}^{1\%}$  the following equations are valid for a given wave length.

$$A + G = T \quad (1)$$

$$AE_a + GE_g + E_0 = E_s \quad (2)$$

where

$T$  = the concentration of protein in g.-%

$A$  = the concentration of albumin in g.-%

$G$  = the concentration of globulin in g.-%

$E_a = E_{1\text{ cm}}^{1\%}$  of albumin

$E_g = E_{1\text{ cm}}^{1\%}$  of globulin

$E_0 = E_{1\text{ cm}}$  (extinction coefficient) of the non-protein substances in serum. This is equal to the optical density of a solution divided by length of solution through which the light passed (1 cm. in this case)

$E_s = E_{1\text{ cm}}$  of serum.

Eliminating  $G$  from the two equations one obtains

$$AE_a + (T - A)E_g + E_0 = E_s \quad (3)$$

From Table II it is evident that most of the non-protein absorption at 2775 Å is due to uric acid and bilirubin. The absorption of an aqueous cholesterol solution is not known. If one assumes that the normal serum uric acid concentration is 4.4 mg.-% (4) and bilirubin 0.5 mg.-%,  $E_0 = 3.1$ . It is seen from Fig. 1 that  $E_a$  is approximately 5.11. However, there are several  $E_g$ 's because there are many globulins. About all that one can do is find an average  $E_g$ . This was accomplished by determining the serum albumin, total protein and serum extinction coefficient and then using the equation

$$5.0 A + (T - A) E_g + 3.1 = E_s \quad (4)$$

For simplicity  $E_a$  was set equal to 5.0 instead of 5.11. It will be seen later that by using this value for  $E_a$  the final equation will have a simple form. Actually any errors resulting from this procedure will probably not be great enough to be very important. The results are shown in Table III.

TABLE III

No	Total protein g.-% (Kjeldahl)	Albumin g.-% (Kjeldahl)	Ext coef 2775 Å, pH 2.0	$E_2$ (calc)
1	8.24	5.30	73.1	14.8
2	7.88	4.91	78.7	17.1
3	7.87	5.35	70.1	16.0
4	7.77	5.25	71.8	16.9
5	7.56	5.30	64.8	15.6
6	7.56	5.25	68.9	17.1
7	7.54	5.00	68.1	15.8
8	7.35	5.11	59.7	13.8
9	7.25	5.15	61.8	15.9
10	7.22	5.01	62.2	15.4
11	7.19	4.89	65.1	16.3
12	7.06	4.86	61.0	15.3
13	7.06	4.80	58.0	13.8
14	6.94	4.64	57.0	13.3
15	6.75	4.86	54.3	14.3
16	6.75	4.79	54.9	14.4
17	6.72	4.69	54.0	13.5
18	6.65	4.45	54.8	13.4
19	6.54	4.57	55.0	14.7
20	6.44	4.52	52.1	13.8
Av.				15.0

It can be seen that the average value for  $E_2$  is 15.0. By substituting this value for  $E_2$  in equation (4) one can write

$$A = 1.5 T - .1 E_2 + .31 \quad (5)$$

The value of  $E_2$  can be set equal to 3.0 with little error. If this is done a simple equation results.

$$A = 1.5 T - .1 E_2 + .3 \quad (6)$$

To test the usefulness of this equation 10 abnormal serums were studied. The total protein and albumin concentrations were obtained from the values of the Kjeldahl and non-protein nitrogen. The albumin and globulin were separated by using sodium sulfate. All of the sera were diluted 1:100 with 0.01 N HCl. Extinction coefficients were determined before and after dialysis for 24 hours. With one exception the values obtained before dialysis were used. The albumin

was calculated by using equation (6) with the value for the total protein concentration as determined from the Kjeldahl nitrogen values. The results are given in Table IV.

TABLE IV

Diagnosis	Total protein g.-% (Kjeldahl)	Albumin g.-% (Kjeldahl)	Non- prot N mg.-%	Ext. coef. before dialysis	Ext. coef. after dialysis	Albumin g.-% (calc.)	Difference in albumin values
Subacute bacterial endocarditis	7.62	2.26	89.0	93.8	92.5	2.31	.05
Lung abscess	7.35	3.01	36.0	85.0	80.6	2.81	.20
Diabetes	6.46	3.90	44.8	59.6	56.8	4.04	.14
Diabetes with uremia	4.67	2.15	71.6	51.1	49.2	2.20	.05
Addison's disease	6.54	4.01	25.2	59.2	57.0	4.18	.17
Cardiac failure	5.95	3.11	64.8	59.0	56.2	3.30	.19
Polycythemia	5.35	3.36	25.4	52.0	50.4	3.12	.24
Pulmonary infarction	7.60	3.92	14.9	74.8	71.9	4.22	.30
Hepatic cirrhosis with ascites	7.10	3.79	30.2	67.2	66.0	4.22	.43
Hepatic cirrhosis with jaundice	5.48	2.51	84.0	75.2	63.3	1.88* (1.00)†	.63

\* Using the value of  $E_s$  after dialysis.

† Using the value of  $E_s$  before dialysis.

It can be seen that, even though there was a marked increase in globulin and decrease in albumin in the two cases with infections, the spectroscopic method gave results comparable to those obtained by the Kjeldahl nitrogen method. Good results were also obtained even when the non-protein nitrogen was 90 mg.-%. Poor results were obtained only from sera of patients with hepatic cirrhosis. This was probably due to the fact that in liver diseases many substances that have ultraviolet absorptions are released into the blood, *e.g.*, tyrosine, phenols, *etc.* In the case of cirrhosis with jaundice there was a marked decrease in the extinction coefficient after dialysis even though bilirubin did not dialyze out. The results were better when the lower value was used.

*Serum Turbidity and Lipemia.* The optical density of only clear solutions can be determined. In cases of lipemia it is necessary to separate the lipid substances from the serum so that a clear solution may be obtained. This usually can be accomplished

by allowing the serum to stand a few days so that the lipids will rise to the surface, leaving a clear solution below. Rapid centrifuging ordinarily will bring about the same result in less time.

*Aging of Sample.* In four normal sera the extinction coefficients were determined immediately after the fresh sera were diluted and after the diluted preparations were allowed to stand at room temperature for one week. None of the changes in the values of the extinction coefficients were greater than 3%. There did not appear to be any changes in the extinction coefficients even if the sera without dilution were allowed to stand in the ice box for several weeks.

*Elimination of the Total Protein Determination.* At first glance it would seem that if the extinction coefficients of serum were determined at two wave lengths, e.g., at 2775 Å (maximum) and 2500 Å (minimum), the total protein concentration would not be needed. However, when this technique was used errors up to 10% were common.

There are several reasons why this technique does not work. In the first place the non-protein constituents of serum have increasing absorptions with decreasing wave lengths and it would be difficult to set up an equation to account for these factors. Also, while the maximum absorptions of the globulin preparations are approximately the same (3) the minimum absorptions are different. This means that it would be difficult to use a single  $E_p$  for 2500 Å.

*Effects of Chemotherapeutic Agents.* Many chemotherapeutic substances have strong absorptions in the ultraviolet. The presence of these substances in serum will sometimes affect the results of the method given here for the determination of albumin and globulin unless special corrections are made. Sulfa drugs when present in serum to the extent of 10 mg.-% should change the results by about 5% (Table II). Penicillin in concentrations of 100 units/ml. has almost no absorption in the ultraviolet. Unfortunately, time did not permit further work to be carried out on this phase of the study.

## EXPERIMENTAL

All measurements were made with a Beckman quartz spectrophotometer with a hydrogen discharge tube and quartz cuvettes 1.00 cm. in thickness. Density readings were made every 25 Å except where maxima or minima occurred, in which case readings were made every 10 Å. The concentration of the solute was adjusted so that the densities of the solutions varied from 0.09 to 0.85. Extinction coefficients were determined by the Beer-Lambert equation.

$$E = \frac{\log \frac{I_0}{I}}{cd}$$

where  $E$  is the extinction coefficient,  $\log I_0/I$  the density,  $c$  the concentration, and  $d$  the depth of the cell. When  $c$  is given in g./100 ml., and  $d$  equals 1 cm., the extinction coefficient is expressed as  $E_{1\%}^{1\text{cm.}}$ . In most cases however the extinction coefficient  $E_{1\text{cm.}}$  instead of  $E_{1\%}^{1\text{cm.}}$  was determined. By  $E_{1\text{cm.}}$  is meant the density of a solution in a cell of 1 cm. thickness. The solute concentrations are expressed separately.

All blood samples were obtained before the subjects had breakfast. The blood was allowed to clot for 1-2 hours at room temperature, and the serum was separated after

centrifugation. Total nitrogen determinations were made on 0.2 ml. serum samples by the micro Kjeldahl method described by Ma and Zuazaga (5). The albumin and globulin were separated by using the sodium sulfate precipitation procedure with 0.5 ml. of serum (6). Nitrogen determinations were made as above. It was assumed that the non-protein nitrogen of each of the sera was 30 mg.-%.

For absorption studies 0.2 ml. of serum as measured with a 0.2 ml. pipet were placed in a 20 ml. volumetric flask and made up to the mark with 0.01 *N* HCl. A few ml. of the diluted serum were placed in quartz cuvettes 1 cm. in thickness and the density at a particular wave length was determined. The density so determined was multiplied by 100 (the dilution factor) to obtain the extinction coefficient  $E_s$  of the serum.

The abnormal sera were studied in a similar manner, but in addition non-protein nitrogen determinations according to the method of Folin-Wu (7) were made. Approximately 10 ml. samples of the diluted serum preparations were dialyzed against 500-600 ml. of 0.01 *N* HCl for 24 hours. Cellophane casings were used, and the containers were tied so that no air space was present to allow dilution. Absorption readings were made before and after dialysis.

## DISCUSSION

To properly evaluate this method for determining serum albumin and globulin, one should compare the accuracy and simplicity of the procedure with those now in use. It is likely that no serum protein determination can be considered to be more accurate than 5%, even though the procedure gives results reproducible within 1%. This fact makes the standardization of any procedure a problem in itself.

The standardization procedure based on Kjeldahl nitrogen values used here is probably considered by most investigators to give the best results. However, this method is actually subject to great error because the various serum proteins contain different amounts of nitrogen (8), and the non-protein nitrogen must be known. The sodium sulfate precipitation technique to separate albumin and globulin is certainly only a crude procedure, and clear-cut separations are not obtained. Protein values as obtained from the Kjeldahl nitrogen and from Tiselius studies often differ by 10%.

After the ultraviolet spectroscopic procedure was standardized with the Kjeldahl nitrogen values, the two methods gave results that were usually in good agreement. However, when the results are not in agreement, as in the cases with cirrhosis, it is difficult to decide which method is more accurate.

In one case of cirrhosis there was a marked decrease in ultraviolet absorption after dialysis. This meant that there were non-protein substances in the serum having appreciable absorption. One would prob-

ably have to dialyze most sera from cases with liver disease because of the presence of tyrosine, phenol, *etc.*, in the blood.

There did not appear to be any effect on the determinations when the non-protein nitrogen was as high as 90 mg.-%. In these cases there was probably but little increase in uric acid concentration.

The bilirubin in normal serum accounts for only a very small fraction of the ultraviolet absorption of serum. In order to affect the results of the method described here by 5% the bilirubin concentration would have to be increased at least 30 times that of normal serum.

If the patient receives chemotherapeutic agents which will affect the ultraviolet absorptions of the serum, the serum should be obtained before the chemotherapeutic agents are given. However, it is possible to draw the blood after the drugs have been administered if the proper correction factors are found.

The simplest way to use the method given here is to determine the total protein concentration by one of the rapid specific gravity methods and then to determine the extinction coefficient for the serum at 2775 Å.

### LABORATORY PROCEDURE

Determine the total protein concentration in the serum sample by one of the rapid specific gravity methods.

Pipet 0.2 ml. of serum into a 20 ml. volumetric flask and dilute up to the mark with 0.01 *N* HCl or water. Determine the density of this solution at 2775 Å using a cell 1 cm. in thickness and a blank of 0.01 *N* HCl or water. Multiply the density by 100 (the dilution factor) to obtain the extinction coefficient  $E_s$  of the serum. Then use the equation

$$A = 1.5 T - .1 E_s + .3$$

The entire procedure should take only a few minutes.

If the serum is from a patient in uremia with a serum non-protein nitrogen greater than 100 mg.-%, or from a patient with liver disease, 10 to 20 ml. of the diluted serum should be dialyzed for 24 hours against 500 to 600 ml. of 0.01 *N* HCl or water before the extinction coefficient is determined. Cellophane casings can be used, but care should be taken to prevent any volume changes.

Only clear solutions can be used. If the diluted serum is turbid, one must let it clear by standing a few days or by centrifuging.

### SUMMARY

The components that account for the ultraviolet absorption of serum were determined.

A simple and rapid method was developed to determine serum albumin and globulin. To use this method the  $E_{1\%}^{1\text{cm}}$  of albumin is set equal to 5.0 and that of globulin equal to 15.0 at 2775 Å.

The method was applied to 10 abnormal sera. Marked increases in globulin and decreases in albumin did not affect the accuracy of the results. Non-protein nitrogen values as high as 90 mg.-% also had no effect. However, in cases of hepatic cirrhosis one must dialyze the serum before making any absorption determinations.

The presence of penicillin in serum will not affect the results, but sulfa drugs in concentration of 10 mg.-% cause approximately a 5% error.

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# The Mutual Precipitation of Proteins and Azoproteins

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## INTRODUCTION

By coupling proteins with diazo compounds a large number of various atomic groups can be introduced into the protein molecule. An almost unlimited number of serologically different azoproteins can thus be prepared (1). Therefore, azoproteins are very important as antigens in immunochemical experiments. Since azoproteins are intensely colored, they can be determined colorimetrically in the presence of antibody proteins, thus rendering possible quantitative determinations of antigen and antibody in the precipitate (2).

The azoproteins chiefly used in serological experiments are albumins or globulins coupled with diazotized amino acids of the formula  $H_2N \cdot C_6H_4 \cdot X$ , where  $X$  is an acid group such as  $-SO_3H$ ,  $-AsO_3H_2$  or  $-COOH$ . Azoproteins containing these acid groups are precipitated by acids, but are soluble in neutral or alkaline media. If solutions of these azoproteins are mixed with solutions of proteins, precipitates are formed in the interisoelectric region, that is, in the pH range between the isoelectric points of the azoprotein and the protein (3).

Conclusions concerning the formation of antibodies *in vitro* have been drawn recently from the precipitation of *p*-azophenyl-arsonic acid ovalbumin by serum globulins (4). We have, therefore, reexamined the mutual precipitation of proteins and azoproteins, applying quantitative methods.

## EXPERIMENTAL

### *Preparation of Proteins and Azoproteins*

Globulin was prepared from beef serum by 0.33 saturation with ammonium sulfate, dissolution of the precipitate in water and reprecipitation. The salts were removed by dialysis and the insoluble euglobulin fraction separated from pseudo-



TABLE I

*Mutual Precipitation of Proteins and Azoovalbumins at Different pH Values*

Each test-tube contained 5 mg. of protein and 5 mg. of azoovalbumin. In exps. 5, 6 and 9 one-fifth of these amounts was used. The figures in the table indicate the ratio of precipitated protein/azoovalbumin in milligrams. Eu=euglobulin, Ps=pseudoglobulin, Sa=Serumalbumin, Ov=Ovalbumin, Lg=Lactoglobulin, As-Ov = *p*-azophenylarsonic acid ovalbumin, Sulf-Ov = *p*-azophenylsulfonic acid ovalbumin.

No. Protein	1 Eu	2 Eu	3 Ps	4 Ps	5 Sa	6 Lg	7 Ov	8 Ov	9 Ov (denat.)
Azoprotein	As-Ov	Sulf-Ov	As-Ov	Sulf-Ov	Sulf-Ov	Sulf-Ov	As-Ov	Sulf-Ov	Sulf-Ov
1	0.0/4.3	0.8/3.6	0.0 4.2	1.0/3.6	0.4/0.4	0.0/0.8	0.6/4.1	1.7/4.2	0.5/0.8
2	0.7/4.2	1.1/2.6	0.3/4.5	1.0/2.7	0.5/0.5	0.1/0.9	0.6/4.3	1.2/4.2	0.5/0.8
3	3.0/4.6	2.9 4.3	0.8/4.5	2.8/3.8	1.0/0.6	0.1/0.8	0.8/4.5	2.3/3.2	1.0/0.9
4	2.7/2.9	2.2, 2.0	1.8/1.7	0.1/1.4	0.0 1.4	0.7/0.9	0	0	1.0/0.4
4.3	2.7/4.5	4.6/3.7	1.7 4.9	2.6/2.7	0.7/0.7	0.7/0.9	0.1/5.0	1.6/3.2	1.0/0.4
4.5	2.7/4.5	3.7 3.7	2.1 4.2	2.7 2.5	0.7/0.7	1.0/0.8	0.2/3.8	0.6/2.5	1.0/0.9
4.7	2.6/4.5	3.7/4.0	3.1/4.2	2.4/2.3	0.6/0.7	0.7/0.8	0.0/0.4	0.9/0.2	1.0/0.9
4.9	2.7/3.7	4.4/3.2	1.8/3.3	2.2/2.1	0/0	0.2/0.5	0.0/0.1	0	1.0/0.9
5.1	2.3/3.2	3.3/3.2	1.2/1.9	1.5/1.6	0	0.4 0.1	trace	0	1.0/0.6
5.3	2.5/2.3	3.0/2.5	0.8/1.0	0.7/0.6	0	0	0	0	1.0/0.45
5.5	1.9/1.9	2.7 1.7	0.3/0.1	0.4/0.1	0	0	0	0	1.0/0.4
5.7	1.7/1.2	2.3/1.4	0	0					1.0/0.4
5.9	1.5/1.0	1.8/1.4	0	0					1.0/0.4
6.1	trace	trace	0	0					trace

\* Citrate-phosphate-borate buffer solution (10) was used at a pH of 2, 3 and 4; acetate buffer solution from pH 4.3 to 6.1. The inhibiting effect of the citrate-phosphate-borate buffer is very distinctly manifest in exps. 7 and 8.

TABLE II

*Precipitation of Euglobulin by p-Azophenylarsonic Acid Ovalbumins Containing Different Amounts of Azophenylarsonic Acid Groups*

(The figures indicate the ratio of euglobulin/azoovalbumin in the precipitate in mg.)

pH	Azoovalbumin		
	A (0.3% As)	B (4.4% As)	C (1.65% As)
5.1	4.4/3.3	3.3/3.3	1.6/1.6
5.3	3.6/2.8	2.7/2.8	1.4/1.4
5.5	3.0/1.8	2.3/2.2	1.2/1.1
5.7	2.5/1.6	2.2/1.8	1.1/0.9
5.9	2.0/0.2	2.1/1.4	0.9/0.1

globulin by centrifuging. Lactoglobulin was prepared from cow's milk (5), ovalbumin from hen's eggs (6) and serum albumin from horse serum (7). A diazo solution prepared in the usual way from 0.45 g. of sulfanilic acid was mixed with 30 ml. of an aqueous solution of 1.35 g. of ovalbumin and 1.8 g. of sodium carbonate (2c). The mixture was kept in the ice box for 3 hours, then dialyzed against distilled water until no dye passed through the cellophane membrane. The azoprotein was precipitated by the addition of dilute HCl and dissolved in water by the necessary minimum of dilute NaOH. The As-ovalbumins (*p*-azo-phenylarsonic acid ovalbumins) A, B and C were prepared similarly by coupling 1.35 g. of the crystalline ovalbumin with 0.17 g., 0.10 g. and 0.025 g. of the diazotized arsanilic acid (*p*-aminophenylarsonic acid), respectively. Analyses of the proteins and azoproteins for nitrogen (Kjeldahl), sulfur (8) and arsenic (9) gave the following results: euglobulin 16.0, pseudoglobulin 14.5, serum albumin 16.1, lactoglobulin 15.6, ovalbumin 15.7 and sulfanilazoovalbumin 14.6% of N; the As-ovalbumins A, B and C contained 10.9, 11.3 and 14.0% of N and 6.3, 4.4 and 1.65% of As, respectively. Solutions containing approximately 1% of the protein or the azoprotein and 1% of NaCl were used in all experiments.

### Precipitation

Five ml. of the buffer solution, 0.5 ml. of the protein and 0.5 ml. of the azoprotein solution were mixed, kept at room temperature for 24 hours and centrifuged. The precipitates were washed with 2.5 ml. of the respective buffer solution and dissolved in 5 ml. of 0.25 *N* NaOH. The amount of precipitated azoprotein was determined colorimetrically in these solutions, solutions of azoovalbumin in 0.25 *N* NaOH being used as standards. The content of the colorimeter cups was rinsed into Kjeldahl flasks for determination of the total nitrogen. The protein nitrogen was obtained as the difference between total and azoprotein nitrogen. One-tenth *N* HCl was used as solvent in the experiments designated by "pH 1" (Table I), citrate-phosphate-borate buffer (10) from pH 2 to 4 and 0.2 *M* acetate buffer solutions at higher pH values (Tables I, II and IV). Some of the experiments were carried out with one-fifth of the amounts mentioned above, that is, with 1.0 ml. of the buffer solution and with 0.1 ml. of the solutions of the protein and azoprotein (Table I, Nos. 5, 6 and 9, Table

TABLE III

#### *Precipitation of Euglobulin by Azoovalbumin in Concentrated Buffer Solutions*

(The figures indicate the ratio globulin/azoovalbumin in the precipitate in mg.)

pH	Protein: Azoprotein:	As-ovalbumin B Molar acetate buffer	$\gamma$ Euglobulin p-Azophenylsulfonic acid ovalbumin Citrate-phosphate- borate buffer (10)
	Solvent:		
4.3		0.72/0.91	0.59/0.24
4.5		0.65/0.85	0.46/0.11
4.7		0.44/0.71	0.41/0.10
4.9		0.35/0.63	0.45/0.11
5.1		0.43/0.28	0.39/0.07
5.3		trace	trace
5.5		0	0

TABLE IV

*Precipitation of Euglobulin by As-ovalbumin A and C at Different Proportions of Both Compounds (pH = 5.5)*

(The figures indicate the ratio euglobulin/azoovalbumin in the precipitate in mg.)

Added		As-ovalbumin A	As-ovalbumin C
Euglobulin	As-ovalbumin		
mg.	mg.	mg./mg.	mg./mg.
9	1	3.1/0.8	1.0/0.3
7	3	3.5/1.8	1.1/0.6
5	5	2.7/1.8	0.8/0.9
3	7	1.6/1.2	0.6/0.8
1	9	0.6/0.3	0.4/0.3

III). Micro colorimetry and micro Kjeldahl methods were employed in these experiments. The denatured (and probably degraded) ovalbumin (Table I, No. 9) was prepared by adjusting a solution of ovalbumin to a pH of 9 by cautious addition of dilute NaOH and keeping it in a boiling water bath for 1 min. The experiments recorded in Table IV were carried out at a pH of 5.5; 5 ml. of the 0.2 *M* acetate buffer solution were mixed with 1% solutions of euglobulin and As-ovalbumin A or C.

## DISCUSSION

The experiments recorded in Table I demonstrate that euglobulin gives precipitates when mixed with azoovalbumins at pH values below 6.1. Since the isoelectric pH of globulins is 6.1 (Tiselius 11), the globulin molecule has a positive net charge in the region of precipitation. Azoproteins, on the other hand, are negatively charged in the pH region of precipitation (3). The precipitation is attributed, accordingly, to the formation of electrovalent bonds (salt-bridges) between anionic groups of the azoprotein ( $-\text{SO}_3^-$ ,  $-\text{AsO}_3\text{H}^-$ ) and cationic groups ( $-\text{NH}_3^+$ ) of the globulin.

The amount of the precipitate is maximal in the interisoelectric region and decreases at low pH values. This is, apparently, due to a reduction of the negative net charge of the azoprotein in acid solutions. In some of the experiments (Table I, Nos. 1, 3 and 6) pure azoovalbumin was precipitated at a pH of 1, no globulin being carried down with the precipitate. The ratio globulin/azoovalbumin increases at higher pH values in accordance with the higher "valency" (negative net charge) of the azoovalbumin and the lower valency (positive net charge) of the globulin at higher pH values.

Similar results were obtained with lactoglobulin, serum albumin and ovalbumin. The isoelectric points of these proteins are 5.2 (12), 4.7 (11) and 5.0 (12), respectively, and they were precipitated by azo-ovalbumins at pH values below their isoelectric points (Table I). Considerable amounts of azoovalbumin were adsorbed, however, on denatured ovalbumin at pH values above 5.3 (Table I, No. 9). It seems, therefore, that soluble compounds between the molecules of the proteins and the azoovalbumin can also be formed above the isoelectric pH. Similarly Putnam and Neurath (13) have observed the formation of complexes between proteins and alkyl sulfonates on the alkaline side of the isoelectric point.

Table II indicates that the number of phenylarsonic acid groups per molecule of azoovalbumin has no distinct influence on the ratio globulin/azoovalbumin in the precipitate. But larger quantities of precipitate were obtained with preparation A (6.3% of As) than with preparation C (1.65% of As). It must be borne in mind, however, that each of the preparations, A and C, is a mixture of azoovalbumins containing different amounts of the phenylarsonic acid groups. It is possible, therefore, that the small amount of precipitate obtained with preparation C is caused by the presence of azoovalbumin molecules containing more than the average content of arsenic.

The precipitation is inhibited by salts. If the 0.2 *M* buffer solution is replaced by a *M* solution of acetate or a 0.34 *M* solution of citrate-phosphate-borate (10), no precipitation of euglobulin occurs above a pH of 5.1 (Table III). This "salting-in" effect of higher concentrations of the buffer is in agreement with the assumption that the precipitation is caused by the formation of salt bridges between ionized groups of the protein and the azoprotein.

The amount of the precipitate and the ratio globulin/azoovalbumin in the precipitate depend on the amount and ratio of both substances in the supernatant liquid. Maximal amounts of precipitate were obtained when a slight excess of euglobulin was used (Table IV). It has also been shown (14) that sulfathiazol-azoproteins precipitate with certain normal and heterologous immune sera.

Pauling and Campbell (4) have observed that *p*-azophenylarsonic acid ovalbumin at a pH of 5.5 is precipitated by serum globulins, which had been kept at 57°C. in the presence of *p*-azophenylarsonic acid resorcinol. No precipitation was observed, however, in neutral solutions. The authors attribute this precipitation to the transformation

of the normal serum globulin into an antibody against the *p*-azophenylarsonic acid group, caused by a rearrangement of the peptide chains at 57°C. It is demonstrated, however, by our experiments (Table I and IV) that *p*-azophenylarsonic acid ovalbumin at a pH of 5.5 is also precipitated by normal euglobulin from beef serum. The claim that antibodies have been formed *in vitro* is not supported by our experiments.

### SUMMARY

Acid azoovalbumins give precipitates with euglobulin, pseudoglobulin, serum albumin, ovalbumin and lactoglobulin in the interisoelectric region. The amount of precipitated protein and azoovalbumin has been determined quantitatively at different pH values and in different buffer solutions. It is concluded that the precipitation is due to the formation of salt-like bonds between negatively charged groups of the azoovalbumin and positively charged groups of the protein.

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(Addendum of the Editors).

# Fermentation of Wood Hydrolyzate by *Fusarium lini* Bolley and Yeast

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## INTRODUCTION

*Fusaria* are one of the unique systems which, with intact living cells, afford the opportunity of drawing conclusions regarding the phase sequence of the enzymatic breakdown of hexoses and pentoses.

Studies during the past 10 years on the mechanism of the degradation by these molds showed, *e.g.*, that: (a) pyruvic acid is formed as the main intermediate in the alcoholic fermentations of hexoses and pentoses (1), bypassing the phosphoglyceric acid (2) step; (b) carbohydrates are abundantly converted into fats; (c) elementary sulfur (3) is utilized as a hydrogen acceptor; and (d) a variety of pigments which can serve as mediators between oxidation and assimilation, and large amounts of thiamine are synthesized in the cells (4)<sup>d</sup>. Furthermore, preliminary data (5) regarding the fermentation of wood hydrolyzates indicated results which, if the aforementioned observations should be properly applied, could be of more interest.

The purpose of the present investigation was, consequently, threefold: (1) establishing the amount of ethanol obtainable from Douglas fir hydrolyzates by fermentation with yeast, and subsequent fermentation of the residual pentoses with *Fusarium lini* Bolley; (2) fermentation of the wood hydrolyzates with the mold alone; and (3) fermentation of the wood hydrolyzates with *Fusarium lini* Bolley plus

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<sup>c</sup> Contribution No. 47.

<sup>d</sup> This paper contains additional pertinent references both to original articles and to reviews.

yeast, it being significant that the loss of pentoses, obtainable from conifer hydrolyzates after yeast fermentation, reaches a surprisingly large figure (4).

Experimental investigations have indicated that two species of microbes may live together to the mutual benefit of both. Usually this is due to the ability of one species to carry out an enzymatic reaction supplementary to the other, and which the other cannot perform of itself. Waksman (6), *e.g.*, has demonstrated that *Trichoderma*, a cellulose organism, which attacks mainly protein in cultures, can develop at the expense of cellulose in the presence of *Rhizopus*. The latter organism utilizes the proteins but cannot attack cellulose.

### METHODS

The total reducing sugar content of the wood hydrolyzate was determined according to Munson and Walker (7); the pentoses by the same method adapted to xylose (8), and the bisulfite method (9). The latter served to measure the furfural content of the hydrolyzate. Ethanol was determined according to Janke and Kropacsy (10) applied on the macro and micro scales. Residual carbohydrates were removed from the nutrient medium by treating with copper sulfate and calcium hydroxide (11). The fungus, *Fusarium lini* Bolley, used throughout this investigation was maintained on a nutrient medium of the following composition: 20.00 g. glucose, 1.00 g.  $\text{KNO}_3$ , 1.50 g.  $\text{KH}_2\text{PO}_4$ , 0.75 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 liter of tap or distilled water. Transfer of the mold was made every 14 days by adding 2 ml. of a spore-mycelial suspension to a flask with a sterile syringe. This suspension had been grown in a 125 ml. Erlenmeyer flask at 28°C. on a solid medium of the same composition as described above supplemented by 20 g. of agar. A solid mat of mycelium was allowed to form and then placed in the hydrolyzate for fermentation of the carbohydrates.

### EXPERIMENTAL

1. *Fermentation of the Wood Hydrolyzates with Yeast.* The sulfuric acid concentration of the original hydrolyzate (12), found to be 0.34%, was neutralized to pH 5.3 with a slurry of calcium hydroxide. The liquid was heated to boiling to lower the solubility of the  $\text{CaSO}_4$  formed and filtered through an asbestos mat.

Lintner and v. Liebig (13), and later Liang (14), made the observation that furfural is reduced to furfuryl alcohol by yeast in the course of an alcoholic fermentation. The fundamental procedure of phytochemical hydrogenation, continued and extended by the investigations of Neuberg, Nord, F. G. Fischer, Mamoli and others (15), indicated that the reducing ability of yeast is connected with some enzymes

operative in hexose fermentation. During the final steps of alcoholic fermentation, cozymase, which is present in yeast in comparatively large concentration, acts as a hydrogen carrier, whereby acetaldehyde is reduced to ethyl alcohol. Furfural, as well as elementary sulfur (3), present in or introduced into the mash, would be a competitor of the acetaldehyde as a hydrogen acceptor and, accordingly, the alcohol production would be diminished. However, if a noncellular hydrogen acceptor, such as furfural, is present, but is reduced *prior* to the fermentation, neither the enzyme system of the yeast nor of *Fusaria* should be markedly affected by the furfural, nor consequently, will alcohol production be influenced. In the present work, sodium metabisulfite (16) was used as the reductor and added to the hydrolyzate in proportion to the furfural content (0.06%) before the fermentation with yeast or *Fusaria* was started.

After pretreatment of the wood hydrolyzate, the nutrient salts were added and the solution divided into diluted (1:5, 3:5) and undiluted portions, half of which were supplemented with the reductor. After sterilization for 20 minutes at  $16 \pm 1$  pounds pressure and inoculation with 3 g. of yeast/100 ml. of hydrolyzate, the media were incubated for 44 hours at 30°C. A sulfuric acid trap was connected to the fermentation flasks and the latter shaken frequently during the incubation period.

The effect of the reductor upon the ethanol production is shown in Table I.

TABLE I  
*Effect of  $\text{Na}_2\text{S}_2\text{O}_5$  on Ethanol Production from Wood Hydrolyzates  
by Yeast Fermentation*

Dilution	pH	Reducing Sugar		Ethanol	
	Final	Initial g./100 ml.	Per cent Fermented <sup>1</sup>	g./100 ml.	Per cent Yield <sup>2</sup>
*1:5	4.5	7.44	59	1.88	83
3:5	4.7	7.44	62	1.98	84
Undiluted	4.3	7.44	52	1.78	89
**1:5	4.8	7.44	65	2.06	83
3:5	4.7	7.44	62	2.12	90
Undiluted	4.6	7.44	54	1.96	94

\* No reductor added.

\*\* Reductor added.

<sup>1</sup> Calculation based on total sugar.

<sup>2</sup> Calculation based on sugar consumed.



2. *Fermentation of Residual Pentoses with Prepared Mats of Fusarium lini* Bolley. After completion of the hexose fermentation with yeast, the mash was filtered through asbestos, concentrated to a low volume *in vacuo* to remove the last traces of alcohol, brought to the original volume with distilled water and treated with 2 g. of Norit/100 ml. of hydrolyzate to remove inhibitory substances other than furfural. Fifty ml. of mash in 125 ml. Erlenmeyer flasks were prepared in triplicate runs. After sterilization, the previously obtained, fully grown fusarial mats were transferred into the mashes. In the preliminary report (5) a 2 ml. spore-mycelial suspension of the mold was used for inoculation, and the alcohol obtained amounted to about 1% of the alcohol derived from a yeast fermentation. In Table II there is recorded the amount of ethanol obtained from the pentose residue by the fermentation with *Fusarium lini* Bolley during sixteen days.

TABLE II  
*Ethanol from Pentose Residue by Fermentation with Fusarium lini*  
*Bolley During 16 Days*

Dilution	Pentose Sugar <sup>1</sup>		Ethanol	
	<i>Initial</i> <i>g./100 ml.</i>	<i>Per cent</i> <i>Fermented</i>	<i>mg</i> /100 ml.	<i>Per cent</i> <i>Yield</i>
* 1:5	2.58	60	408	88]
3:5	2.58	46	161	37
Undiluted	2.60	45	234	69
** 1:5	2.56	63	384	77
3:5	2.43	65	210	48
Undiluted	2.58	61	139	34

\* No reductor added.

\*\* Reductor added.

<sup>1</sup> Calculated as xylose.

In Fig. 1 are presented the changes in pH of the wood hydrolyzate media during a 16 day fermentation with *Fusaria*. The rapid rise in pH after the 12th day, accompanied by a marked falling off in mycelial weight (see Fig. 2) gives considerable evidence of autolysis taking place as well as elaboration of nitrogen-containing products in the media from decomposition of the mold.

3. *Determination of Ethanol obtainable from Wood Hydrolyzate with Fusaria Alone.* The mash was prepared as described above for the pentose residue, and experiments set up with the usual 50 ml. volume of wood hydrolyzate in 125 ml. Erlenmeyer flasks and allowed to incubate for 11 days at 28°C. The result of the analyses made on the 3rd, 5th, 7th, 9th and 11th days are presented in Table III.

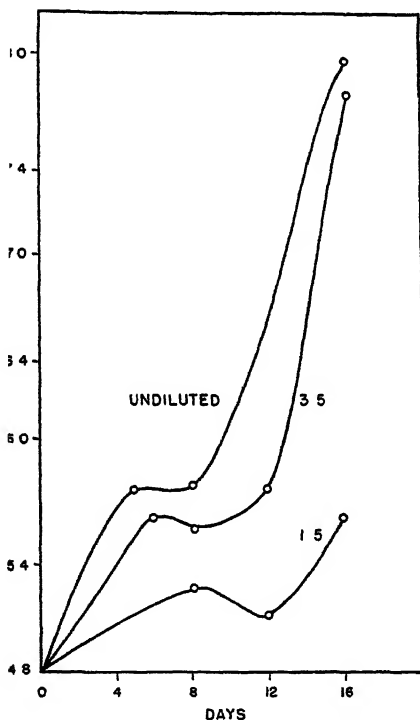


FIG. 1. Change in pH during a 16 Day Growth of *Fusarium lini* Bolley on Douglas Fir Hydrolyzate.

4. *Ethanol from Wood Hydrolyzate by Fermentation with Fusarium lini Bolley plus Yeast.* This part of the investigation was approached in two ways: (1) by adding yeast (3 g.-%) two days prior to analysis of the hydrolyzate; (2) by simultaneous fermentation of the hydrolyzate by *Fusaria* plus yeast. The data are compared and recorded in Fig. 3.

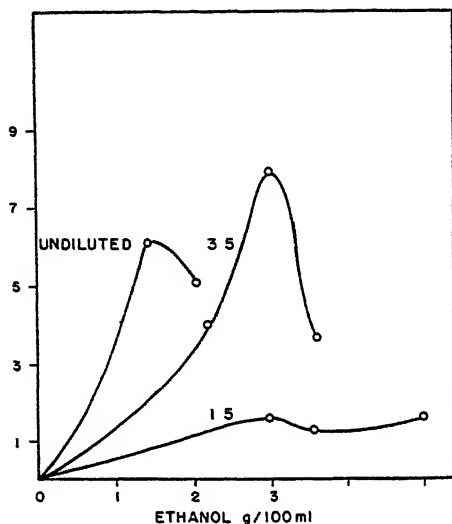


FIG. 2. Ethanol Accumulation and Mycelium Growth during Fermentation of Residual Pentoses with *Fusarium lini* Bolley.

TABLE III

*Ethanol Obtained from the Fermentation of Wood Hydrolyzate by Fermentation with Fusarium lini Bolley Alone*

Day	pH	Reducing Sugar		Ethanol	
	Final	Initial g./100 ml.	Per cent Fermented	g./100 ml.	Per cent Yield
*3	4.8	7.22	28	0.40	38
5	5.5	7.22	60	1.35	61
7	5.8	7.22	73	1.61	59
9	5.6	7.22	84	1.48	47
11	6.0	7.22	88	1.50	46
**3	4.8	7.22	25	0.54	59
5	5.4	7.22	62	1.46	64
7	5.9	7.22	73	1.76	65
9	5.9	7.22	87	1.63	50
11	5.7	7.22	89	1.53	46

\* No reductor added.

\*\* Reductor added.

The first curve (I) shows a decrease of the alcohol accumulated after the peak is reached. This is due to the dehydrogenation of the alcohol formed by the mold (17). The trend of the other curves (II and III) is basically similar. The rise in these last two curves shows the onset of the pentose fermentation by the *Fusaria*. When the yeast was introduced into the mash on the 3rd day, the fermentation by the mold had not progressed very far, judging from previous observations of the

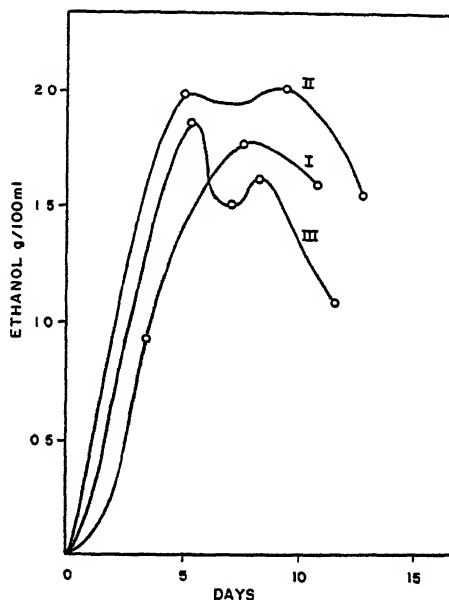


FIG. 3. I. Ethanol by *Fusarium lini* Bolley Fermentation. II. Ethanol by *Fusarium lini* Bolley Fermentation with Yeast Added 2 Days before Analysis. III. Ethanol by *Fusarium lini* Bolley Fermentation plus Yeast.

rate of action on sugar media. The value obtained when the yeast alone fermented the carbohydrates of the hydrolyzate totaled about 2 g. of ethanol/100 ml. of hydrolyzate. Here the value is somewhat lower. If the pentose had been attacked by the *Fusaria* first, and the hexose later attacked by the yeast when it was introduced into the mash, the sum total of alcohol produced by both fungi would have been higher than is recorded. From this observation, it may be supposed that the mold initiated the fermentation of the hexoses and

pentoses, and the enzymatically much more saturated yeast completed the degradation. Another explanation may be that of diffusion. The latter is a controlling factor (18) in all cell fermentations. Sobotka *et al.* (19) reported that glucose fermentation is retarded in the presence of xylose or arabinose. This inhibitory "pentose" effect runs parallel to the ratio of the hexose and pentose content. These investigators studied the speed of diffusion of various sugars into the yeast cell and found that xylose diffuses into the cell slightly sooner than glucose. Applying this observation to the hydrolyzate fermentation, the lower ethanol values obtained in the 'symbiotic' fermentation by means of the two fungi may have been due to a competitive diffusion which slowed down alcohol accumulation.

### DISCUSSION

As acid hydrolyzates of wood contain extractives, sugar decomposition products and furfural, which inhibit ethanol formation, the wort was prepared for fermentation with yeast and *Fusaria* by neutralization, application of heat and addition of sodium metabisulfite as a reductor. Whereas a steady increase in alcohol derived from yeast fermentation is observed on the solutions containing the reductor, no such increase was noticeable during the pentose fermentation with *Fusarium lini* Bolley. It would, therefore, follow that either all the residual inhibitory substances had been deactivated by the pretreatment of the hydrolyzate, or the mold was insensitive to their effect, or the rate of dehydrogenation of the alcohol is more rapid than the rate of fermentation diminished by the action of some unchanged inhibitors. Moreover, the fungus possesses a powerful dehydrogenase system, which enables it to utilize ethyl alcohol as a carbon source when the sugar content of the medium is nearly exhausted, dehydrogenating the accumulated alcohol.

Determination of the change in pH during the mycelial development indicated that the optimum growth occurs at the peak of the alcohol accumulation. It appears that this rugged fungus possesses the ability to adjust its inner pH (20) to favor better growth, so that, during the slower degradation of the pentoses, the mold may also have utilized the acids formed, thus giving rise to an increase in pH.

The "pentose" alcohol found after fermentation of the pentose residue with *Fusaria* varied up to amounts of 19% of the alcohol

obtained from the hexoses by yeast. This increase, as compared with the 1% obtained by inoculation with spore-mycelial suspension, is marked, and is probably due to (a) the application of prepared mats of *Fusaria* and (b) the reduction of the furfural present. Furthermore, this increase surpasses by 7-13% the surplus "Pentose" alcohol obtained with *Fusaria* from sulfuric acid-hydrolyzed wheat mash (21).

From the recorded results of the "symbiotic" fermentation with yeast plus *Fusaria* it appears that each fungus carried out its own degradation independently of the other. It is true that there is a lower alcohol accumulation when both fungi are added to the media simultaneously as well as when the yeast is added two days prior to each analysis, but this difference is also probably due to dehydrogenation of the accumulated alcohol or to an inhibitory "pentose" effect.

It can be recognized, therefore, that pentose-containing wastes can be utilized by fermentation with *Fusaria* and also that the yeast-unfermented Douglas fir hydrolyzates can yield, by fermentation with the mold alone, ethyl alcohol in quantities only a little lower than those obtained from the yeast fermentation of the hydrolyzate.

#### ACKNOWLEDGMENTS

The cultures of *Fusarium lini* Bolley used in these investigations were originally obtained from the Biologische Reichsanstalt, Berlin-Dahlem, through the courtesy of Dr. H. Wollenweber; the compressed bakers' yeast through the courtesy of the National Grain Yeast Corporation, Belleville, N. J.; the Douglas fir hydrolyzates through the courtesy of Dr. E. E. Harris of the U. S. Forest Products Laboratory, Madison, Wis.

#### SUMMARY

1. The mold, *Fusarium lini* Bolley, ferments hexoses and pentoses present in Douglas fir hydrolyzates to ethyl alcohol.
2. Prior to the fermentation with yeast or *Fusaria*, the inhibitory furfural present in the hydrolyzate was reduced to furfuryl alcohol by adding sodium metabisulfite as a reductor. Increased alcohol accumulation was observed when the hydrolyzates were fermented with yeast after the addition of the reductor.
3. Yeast fermentation of the Douglas fir hydrolyzates gave 89% yield of ethanol. The subsequent fermentation of the pentose residue with prepared mats of *Fusarium lini* Bolley gave yields of up to 77%, which represent up to 19% of the alcohol obtained from the hexoses

by yeast. Fermentation with *Fusarium lini* Bolley alone showed a yield of 65%, a value which was also recorded by the "symbiotic" fermentation of the two fungi.

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# Micro Determination of Pentoses by the Bisulfite Method

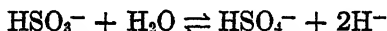
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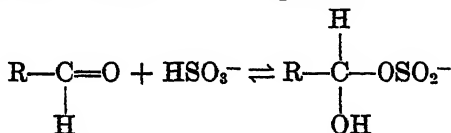
## INTRODUCTION

The bisulfite method for the determination of aldehydes, first proposed by Ripper (1), applied to pentoses by Jolles (2), and modified by Youngburg (3), Meissner (4) and others, has been the subject of thorough investigations (5, 6, 7, 8, 9). The method consists in converting the pentose sugars to furfural by heating with 85%  $\text{H}_3\text{PO}_4$  and steam distilling the product into a solution of  $\text{NaHSO}_3$ . After formation of the furfural addition product, the excess bisulfite is oxidized with a standard iodine solution, using starch indicator, according to the following equation:



The blue color may be discharged by addition of a few ml. of a saturated solution of  $\text{NaHCO}_3$ . This addition of mild alkali causes a dissociation of the furfural-bisulfite compound and the freed salt may be titrated with a standard iodine solution to the reappearance of the blue color.

The accuracy of the method depends upon the constancy of the equilibrium point as indicated in the equation:



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The equilibrium is controlled (10, 11, 12) by the dissociation of the bisulfite solution, the furfural-bisulfite solution, the  $H^+$  concentration of the distillate and the reaction temperature. The addition compound has a  $K$  of about  $1 \times 10^{-4}$ . The dissociation of this compound can be decreased by lowering the temperature, although the time required for establishment of equilibrium is greatly increased. Increasing the concentration of both the furfural and the bisulfite, increases  $K$  and permits better analytical values. However, unless a temperature of about  $15^\circ C.$  is maintained during the preparation of the addition compound, an error will be introduced due to the volatility of the bisulfite solution.

In the course of the investigations on molds carried out in this laboratory, we availed ourselves of micro analytical procedures for the determination of succinic acid (13) and glycerol (14), and the above mentioned controlling factors: *i. e.*, temperature, pH and concentrations, were studied, at the suggestion of Dr. Nord, to find out whether this method might be used for the micro determinations of *d*-xylose and *d*-ribose. When determinations were made *with* distillations of furfural, samples containing 10 mg. of pentose sugar proved to be the best minimum amount for accurate analysis. But a large number of determinations were finally and successfully carried out using 5 mg. or less in the sample, *without* distilling the furfural. However, the micro method could not be adapted, thus far, for determinations of pentoses in such products as wood hydrolyzate (15).

## EXPERIMENTAL

### 1. Chemicals

0.950 g. of anhydrous c. p. metabisulfite were diluted with distilled water to 1 liter to prepare the 0.01 *N* bisulfite solution. The 0.01 *N* iodine was standardized with 0.01 *N* thiosulfate, using starch indicator. This solution was freshly prepared before each series of experiments and frequently checked. Freshly distilled aniline, free from furfural, was used to prepare the aniline acetate test paper. *d*-Xylose  $[\alpha]_D^{25} = -19.1^\circ$  was obtained from A. D. Mackay, New York, and *d*-ribose  $[\alpha]_D^{25} = -19.0^\circ$  from Dr. Georg Henning, Ltd., Berlin-Tempelhof.

### 2. Procedure

*a. Pentose Determination with Distillation of the Furfural.* Three ml. of pentose solution containing 10–30 mg. of sugar are pipetted into a test tube and 10 ml. of  $H_3PO_4$  added. The solution is heated in an electrically controlled asbestos mantle to  $125^\circ C.$ , then connected to a steam generator and steam-distilled into an ice-cooled receiver. During distillation of the furfural the temperature of the mantle is allowed

to reach 175°C., but not higher, and the distillation is continued until aniline acetate paper fails to give a red color. After distillation is complete, the solution is diluted to a definite volume, usually 200 ml., and aliquots are treated with an excess of 0.02 *N* bisulfite solution. A solution of 0.01 *N* iodine solution with starch indicator is used for the oxidation of the bisulfite. After the first end point, the blue color is discharged with a few ml. of a saturated solution of NaHCO<sub>3</sub>, and the freed bisulfite titrated with iodine to the reappearance of the blue color. If a blank is run, a check may be made on the first end point, but the same conditions of temperature, pH and concentrations must be rigidly observed.

*b. Pentose Determination without Distilling the Furfural.* One ml. of a solution containing 500  $\gamma$ —5 mg. of pentose sugar is placed in a flask connected to a small reflux condenser and 2 ml. of 85% H<sub>3</sub>PO<sub>4</sub> added. The temperature of the mantle is maintained at about 130°C. for 10 minutes, the contents allowed to cool, diluted to the 100 ml. mark and aliquots taken for analysis. These are chilled and neutralized to methyl orange. The aliquots are treated with an excess of the bisulfite solution and allowed to stand about two hours at room temperature to assure complete formation of the furfural-bisulfite compound. A 0.01 *N* bisulfite solution is used for the preparation of the addition compound and a 0.005 *N* iodine for the titration. A buret graduated in 0.05 ml. divisions gave very accurate end points. The first end point was recorded.

### Sample Calculation

A sample of *d*-xylose converted to furfural by heating with 85% H<sub>3</sub>PO<sub>4</sub> is steam-distilled into 25 ml. of 0.02 *N* sodium metabisulfite. Titrating with 0.0096 *N* iodine, the second end point gave 10.40 ml. of iodine used to react with the freed bisulfite.

ml. iodine used  $\times$  normality of iodine  $\times$  75 = mg. pentose. 10.40 ml. iodine  $\times$  0.0096 *N* iodine  $\times$  75 = 7.47 mg. *d*-xylose. Using the factor 2, the recovery in mg. equals 14.94, which represents 99.6% recovery of pentose sugar.

Fig. 1 shows the quantity of *d*-xylose that can be accounted for by varying the volume of the 85% H<sub>3</sub>PO<sub>4</sub>, omitting the distillation of the furfural.

In Tables I and II are recorded the values of the *d*-xylose and *d*-ribose determinations *with* and *without* distillation of the furfural formed.

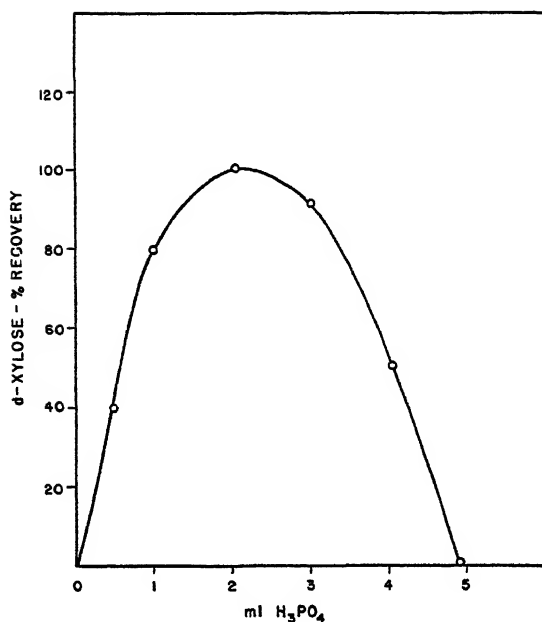


FIG. 1. *Per cent Recovery of d-Xylose (5 mg./ml.) Showing Number of ml. of H<sub>3</sub>PO<sub>4</sub> Required; without Distillation of Furfural.*

TABLE I

*Determination of d-Xylose and d-Ribose by the Bisulfite Method with Distillation of the Furfural*

Number of Determinations			Pentose Recovery	Sugar Recovery	Standard Deviation in Per cent
	ml.	mg.	mg.	Per cent	
<i>d-Xylose</i>					
25	2	10	4.64	94	+0.01
6	10	50	23.00	92	-0.01
<i>d-Ribose</i>					
12	10	10	4.84	96	-0.02
6	4	20	9.89	98	-0.02
3	10	40	19.50	97	0

TABLE II  
*Determination of d-Xylose and d-Ribose by the Bisulfite  
 Method without Distillation of the Furfural*

Number of Determinations			Pentose Recovery	Sugar Recovery	Standard Deviation in Per cent
	ml.	mg.	mg	Per cent	
<i>d</i> -Xylose					
6	1	0.50	0.22	86	-1.4
7	1	1.00	0.46	94	-0.01
6	0.5	2.50	1.23	98	+1.4
3	2	4.00	1.90	95	0
23	1	5.00	2.50	100	-0.7
<i>d</i> -Ribose					
2	1	1.00	0.50	100	+1.0
2	0.25	1.25	0.55	88	-1.4
6	0.50	2.50	1.23	98	+0.4
17	1.00	5.00	2.36	94	-0.6

## CONCLUSION

From the data presented, it can be seen that samples of 500  $\gamma$ -5 mg. of *d*-xylose or *d*-ribose/ml., without distillation of the furfural, gave, for a number of determinations, an average of 95% recovery. Using the micro method, the time requirement is two hours. It can be advantageously applied in cases in which a slight acid concentration will not interfere with the determinations.

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**Ascorbic Acid, Peroxidase and the Protease "Mexicain,"  
in the Latex of *Pileus Mexicanus***

Investigations on the biochemistry of latex, have led us to find, in addition to Mexicain (a proteolytic enzyme studied in this Laboratory (1)), relatively high concentrations of ascorbic acid and peroxidase.

Sumner and Howell (2) had already reported peroxidase activities in a mixture of latices from several species of *Phycus*, but without specifying which they were.

We have been unable to find any reference to the presence of ascorbic acid in plant latex, either free or associated with a peroxidase or proteolytic enzymes.

That proteolytic enzymes are activated *in vitro* by ascorbic acid has been shown by Karrer and co-workers (3), Maschmann and Helmert (4) and Purr (5).

According to Tauber (6) there are interrelations between ascorbic acid and peroxidase in the biological phenomena of oxidation-reduction.

In securing the new data we proceeded as follows: peroxidase activity was measured by the purpurogallin method of Willstätter and Stoll (7), modified only by the use of one-tenth quantities of reagents (8). Ascorbic acid was determined by the method of Roe and Oesterling (9); iron by the Stugart method (10), and nitrogen, by micro Kjeldahl.

Results in the following table are consistent in a series of 10 similar determinations.

With *dl*-alanine and hydrogen peroxide solution (11), considered specific for ascorbic acid with amines and not duplicated by any other reducing substance, latex gave a reddish color.

Three ml. of a 1% solution of fresh latex plus 0.2 ml. of the reagents commonly used to test peroxidase activity qualitatively (benzidine, pyrogallol, guaiacol, hydroquinone, catechol, *p*-phenylenediamine, adrenaline and tyrosine) plus 0.2 ml. of 0.5%  $H_2O_2$ , gave strongly

TABLE I

*Purpurogallin Number and Ascorbic Acid, Dehydroascorbic Acid, Nitrogen and Iron in the Latex of Unripe, Fullgrown Fruits of Pileus mexicanus*

Purpurogallin number 50 mg. of fresh latex in 200 ml. of substrate	Data obtained with 100 g. of fresh latex				
	Ascorbic acid	Dehydroascorbic acid	N <sub>2</sub>	Fe+++	Moisture
0.73	mg. 73.0	mg. 0.0	g. 2.14	mg. 0.58	g. 77.8

positive results; with *o*-, *m*- and *p*-cresol, results were only slightly positive.

What has greatly impressed us is the constant association we have found in the latex of *Pileus mexicanus* of peroxidase activity together with a high ascorbic acid content.

As latex is a product of great physiological activity, we are considering the many possible functional correlations existing between protease, ascorbic acid and peroxidase as a system, and are investigating these subjects further.

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### Phytofluene in *Neurospora*

As reported before (1), phytofluene is a colorless  $C_{40}$ -polyene, with a higher degree of hydrogenation than carotene. Its regular occurrence in Phanerogames has been demonstrated, and it was shown that its biosynthesis runs parallel with the formation of polyene pigments in *Pyracantha angustifolia* berries. It seemed advisable to delineate the border lines of the occurrence of phytofluene in the Cryptogames as well. It was reported (2) that a strain of *Rhodotorula rubra* and some of its colored mutants contained 0.6–1.3 mg. of phytofluene/100 g. of dehydrated yeast. In our *Neurospora* cultures phytofluene occurs in higher quantities than in the red yeast, viz., 3–4.5 mg./100 g. of dry material.

Since, at the present time, phytofluene is the only well characterized compound which could be reasonably taken into consideration as a biological precursor of carotenoid pigments, some experiments were undertaken to establish the ratio, pigment/phytofluene, in *Neurospora*, the pigmentation of which is stimulated by light (Went (3)).

The mycelium of our starting material, when grown in darkness, showed a pale orange color. Exposure of such cultures to white light for a day resulted in a visible intensification of the pigmentation. Extinction values of the total pigment showed a roughly twofold increase. Cultures which had been illuminated continuously for the total growth period were bright reddish-orange, and their pigment content was about 4 times greater than that of parallel cultures grown in the dark.

The phytofluene content was found to be as follows: dark, 3.2 mg.; light, 2.9 mg.; and dark to light, 0.8 mg./100 g. of dry mold. The decrease in phytofluene upon illumination of cultures grown in darkness could possibly mean a conversion into pigment.

When the same strain was used for mass cultures, the visible difference in pigmentation between "light" and "dark" mycelia was, for unknown reasons, noticeably less than in the experiment just described. Furthermore, no color increase was apparent upon exposure of the fully developed "dark" cultures to light. The total pigment quantity was estimated by summation of the individual pigments, each of which had been determined spectrophotometrically, after chromatographic separation. The following values denote mg. of polyene/100 g. of dry mold.



<i>Neurospora</i>	Grown in darkness	Continuously illuminated during growth	After the same growth period in darkness, illuminated for a day
Sum of carotenoid pigments	16.9	30.0	14.3
Phytofluene	3.7	3.0	3.2

Evidently, the absolute amounts of phytofluene present were practically independent of the illumination, while the biosynthesis of the colored polyenes was markedly stimulated by continuous illumination during the growth period. The ratio, pigment/phytofluene, changed in a manner which is entirely different from that observed in ripening *Pyracantha*, in which this ratio remained practically constant although the total polyenes increased more than twofold (1).

It is planned to extend such studies to strains which are more photosensitive than the one used for this investigation, and also to study the nature and formation of polyenes in *Neurospora* mutants. The composition of the pigment will be described by one of the authors (F. H.).

### EXPERIMENTAL

The mold was grown at 30–31°C. for two weeks in 2 l. Fernbach flasks containing the so-called "complete" agar medium (4) supplemented with additional yeast extract, malt extract and sucrose. Illumination was provided by Mazda "daylite" fluorescent lamps (40 Watt) set at an angle over the cultures at a distance of 15–20 cm. from the agar surface.

*Estimation of Phytofluene.* The mycelium was separated from the agar, coarsely fragmented in a meat grinder and dehydrated by storing overnight under acetone. The solid residue was then ground under acetone in a mortar and extracted further by mechanical shaking in the presence of fresh acetone, until the last portion of the extract was only faintly colored. The polyenes were transferred to petroleum ether (b.p. 60–70°C.) by the addition of water. The pigment solution was saponified and further treated as described earlier (1). The phytofluene was easily recognized by its maxima and minima as determined with a Beckman spectrophotometer (367, 358–9, 348, 338, 332 m $\mu$ , in petroleum ether), and estimated quantitatively in the same solvent on the reported basis,  $E_{1\%}^{1\text{cm.}} = 1200$  at 348 m $\mu$ .

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### The Microbiological Determination of Tryptophan in Unhydrolyzed Casein with *Tetrahymena Geleii* H \*

Sirs:

Tryptophan has been determined in intact (unhydrolyzed casein) with *Tetrahymena geleii* H,<sup>a</sup> a strain of ciliated protozoa. The authors' interest in this problem was stimulated by the findings of Kidder and Dewey (1-3) that tryptophan is essential for the growth of *Tetrahymena* on an amino acid-containing medium and that these organisms utilize unhydrolyzed proteins.

A basal medium essentially of the composition described by Kidder and Dewey (3), and the microbiological techniques commonly used in the authors' laboratory for the determination of amino acids with lactic acid bacteria, were employed. A sample of purified casein,<sup>b</sup> dispersed in water at pH 7, was added in graduated concentrations to a series of tubes each containing the basal medium and water in a total volume of 10 ml. A standard was prepared similarly using *l*-tryptophan in place of casein. The tubes were autoclaved, inoculated with a uniform washed suspension of *T. geleii* H cells<sup>c</sup> and incubated 9 days at 25°C. The density of the cell suspensions was determined with a photoelectric colorimeter.

The results of an assay of casein are given in Table I.

The tryptophan content of casein was found to be 1.3% (uncorrected) in the present experiments. This value lies within the range,

\* This work was aided by a grant from the National Institute of Health, U. S. Public Health Service.

<sup>a</sup> Obtained through the courtesy of Dr. George W. Kidder.

<sup>b</sup> Sample same as that referred to by Dunn *et al.* (4).

<sup>c</sup> Grown on an inoculum medium containing 2% proteose-peptone (Difco) and 1% glucose.



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# Fibrinolysin: Nomenclature, Unit, Assay, Preparation and Properties

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## INTRODUCTION

An historical background for fibrinolysin and streptokinase was presented by Kaplan (1) very recently. In a footnote he briefly discussed the inadequacy of the nomenclature for compounds related to the lytic phase of blood coagulation and recommended that steps be taken to clarify the confused terminology. We are in accord and suggest the following names for the compounds:

1. *Fibrinolysin*—the activated lytic principle from serum or plasma. This enzyme is primarily concerned with dissolving fibrin and changing fibrinogen so that it will not clot with thrombin.
2. *Profibrinolysin*—the inactive form or precursor of fibrinolysin. This compound is the proenzyme from serum or plasma activated by streptokinase, organic solvents and other enzyme activators. We suggest profibrinolysin to conform with the prothrombin  $\rightarrow$  thrombin enzyme system in coagulation.
3. *Streptokinase*—the exotoxin produced by certain strains of hemolytic streptococci. This is the streptococcal activator of Tillett and Garner (2). It was the misnaming of this kinase that has resulted in the current confusion.
4. *Antifibrinolysin*—fibrinolysin inhibitor, the naturally occurring plasma or serum compound(s) that blocks the action of fibrinolysin on fibrin or fibrinogen.
5. *Antiprofibrinolysin*—profibrinolysin inhibitor, the naturally occurring plasma or serum compound(s) that blocks the conversion of profibrinolysin to fibrinolysin.

6. *Antistreptokinase*—streptokinase inhibitor, the naturally occurring plasma or serum compound(s) that inhibits streptokinase (3). These may be developed during the immunological response to streptococcal infection.

These names, as defined, will be used by us in this publication.

## UNIT AND ASSAY

Since there is no standard set unit of activity for fibrinolysin we have defined our own which is as follows: One unit of fibrinolysin is that amount which will dissolve 1 ml. of a 0.3% fibrin clot in 120 seconds at pH 7.2 and 45°C. in an isotonic saline system buffered with imidazole (4).

*Assay.* Two-tenths ml. of a saline solution of fibrinolysin of known concentration was added to 0.1 ml. of 100 unit/ml. thrombin solution in 50% glycerol in a 10 × 75 mm. test tube. Three-tenths ml. of a 0.6% fibrinogen solution containing imidazole buffer was then blown into the tube as a stopwatch was simultaneously started. The tube was placed in a water bath at 45°C., removed every 15 seconds and tilted gently. The endpoint of the assay was considered to be the earliest time the solution flowed on gentle tilting of the tube.

## PREPARATIONS

Early preparations of fibrinolysin were found to clot fibrinogen (5,6). Some authors have attributed this property to the enzyme itself, but it has recently been shown that clotting properties, either direct or indirect through activation of prothrombin, cannot be ascribed to fibrinolysin (7). In order to prepare prothrombin- and thrombin-free fibrinolysin, the prothrombin was removed from bovine and human plasma by adsorption on an excess of  $Mg(OH)_2$  cream and by centrifugation to remove the adsorbate. The profibrinolysin was then concentrated by fractionation and precipitation techniques and activated.

An alternate procedure, which gave a far superior fibrinolysin product, was the fractionation of profibrinolysin from serum by essentially the same method.

*Preparation of Serum.* Serum was prepared from oxalated bovine or human plasma, collected as described previously (8), by the addition of 0.3% excess calcium chloride to the vigorously agitated plasma. The fibrin precipitated in strands. Stirring was continued for 30 minutes after the fibrin separated, to allow for complete conversion of prothrombin to thrombin and for antithrombin (present in the serum) to destroy the thrombin.

Each lot of serum was tested in the following ways:

1. One ml. serum + 0.1 ml. 100 U. thrombin → no clot. Therefore all fibrinogen was removed.
2. Two-tenth ml. serum + 1 ml. purified fibrinogen → no clot. Therefore all thrombin destroyed.

3. One ml. serum + 1 ml. purified fibrinogen + 0.2 ml. purified lung extract (4) → no clot. Therefore all prothrombin was converted to thrombin and destroyed as proved in 2.

*Preparation of Fibrinolysin.* Plasma<sup>1</sup> treated with  $Mg(OH)_2$  or serum (which is preferred since it yields fibrinolysin of higher purity) was cooled to 5°C., a saturated solution of  $(NH_4)_2SO_4$  added dropwise, with constant stirring, to 25% of saturation. The precipitated proteins were removed by centrifugation for 3 minutes at 5000 r.p.m. The solids were discarded. The supernatant solution was cooled to 0°C. and the degree of  $(NH_4)_2SO_4$  saturation increased to 29% by the further dropwise addition of saturated  $(NH_4)_2SO_4$  with constant stirring. The precipitate was collected by centrifugation at 5000 r.p.m. for 3 minutes. The supernatant solution was discarded.

The precipitate was dissolved in 100 ml. distilled water, transferred to a separatory funnel and shaken intermittently for 30 minutes with 25 ml.  $CHCl_3$ . After this activation, the  $CHCl_3$  layer was separated and discarded. The aqueous phase was dialyzed for 16 hours against cold running tap water in Visking "No Jax" casings. The precipitate which formed on dialysis was collected by a short fast centrifugation, as above, dissolved in 100 ml. of physiological saline, diluted to 1500 ml. with cold distilled water (to a specific resistance of the solution—approximately 400 ohms), cooled to 0°C. and adjusted to pH 5.5 (glass electrode) with  $N$  HCl. The precipitate which formed was collected by a short, fast centrifugation, dissolved in 100 ml. physiological saline, adjusted to pH 7.0 to 7.2 with 0.1  $N$  NaOH, shell frozen and lyophilized.

## DISCUSSION

Fibrinolysin was also prepared according to Christensen (9) and activated with streptokinase (10). The activity of this preparation was 0.02 U./mg. of lyophilized solids, or 0.38 U./mg. nitrogen. Since it appeared questionable whether this method of preparation yielded maximum results,  $CHCl_3$  activation of a sample of Christensen's "lysin factor" (profibrinolysin) was accomplished as follows:

72 ml. of Christensen's "lysin factor" were placed in a separatory funnel, 20 ml.  $CHCl_3$  added and the two phases mixed by intermittent shaking for 90 minutes. The  $CHCl_3$  layer was separated and discarded. The aqueous phase was dialyzed for 16 hours in Visking "No Jax" casings against cold running tap water; the precipitate which formed on dialysis was collected by centrifugation, dissolved in 45 ml. physiological saline, adjusted to pH 7.15 with 0.1  $N$  NaOH, shell frozen in a round bottom flask and lyophilized. This solid assayed 0.13 U./mg. or 1.44 U./mg. nitrogen.

<sup>1</sup> Four l. of beef plasma were treated with 600 ml.  $Mg(OH)_2$  cream (8). The  $Mg(OH)_2$  with adsorbed prothrombin was removed from the supernatant plasma by centrifugation for 10 minutes at 5000 r.p.m.

## EVALUATION OF DATA AND PROPERTIES OF FIBRINOLYSIN

Table I gives the assay results of a few of the fibrinolysin preparations from plasma. It is quite evident that these products surpass earlier preparations in activity and purity.

TABLE I  
*Preparations of Fibrinolysin from Plasma*

No.	Nitrogen	U./mg.	U./mg. Nitrogen
	<i>per cent</i>		
115	12	0.28	2.25
205	8	0.20	2.60
220	4	0.12	3.00
128	9	0.37	4.11
112	3	0.23	7.19
005	6	0.03	4.75

Table II shows the remarkable improvement in purity of the fibrinolysin when prepared from serum. When these data are compared with the unit activity of other preparations an increase of 6000% or more becomes apparent.

TABLE II  
*Preparations of Fibrinolysin from Serum*

No.	Nitrogen	U./mg.	U./mg. Nitrogen
	<i>per cent</i>		
205	11	2.03	18.45
206	8	2.07	25.89
207	11	2.03	18.45
211	10	2.03	20.30
212	10	1.42	14.20
304	8	1.77	22.13
305	11	2.53	23.00
306	9	2.54	28.22

## PROPERTIES

Tagnon (11) and Kaplan (12) discussed some of the properties of fibrinolysin, but not all the characteristics they ascribed to this enzyme still hold true for the more refined product. Our observations have

shown that fibrinolysin is an euglobulin-water insoluble, saline soluble nondialyzable protein enzyme. Its point of minimum solubility is near pH 5.5. Prothrombin does not inhibit fibrinolysin; but rather this enzyme destroys prothrombin. Thrombin is immune to the proteolytic action of fibrinolysin. Fibrinolysin destroys fibrinogen and fibrin, splitting each of these molecules into 2 (18) or more parts:  $\alpha$  and  $\beta$  fibrin and  $\alpha$  and  $\beta$  fibrinogen derivatives. Fibrinolysin does not clot fibrinogen.

### SUMMARY

A simple system for naming the compounds related to the lytic phase of blood coagulation is suggested. The unit of fibrinolysin is defined and an assay procedure presented. Methods of preparation of fibrinolysin, comparison of various products and the corrected properties of the enzyme are given.

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# Chemical Inactivation of Streptomycin

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## INTRODUCTION

The problem of inactivating streptomycin is of interest in the development of a suitable sterility test as well as in the study of the mode of action and chemical nature of this antibiotic.

A preliminary report on the effect of cysteine, 2-aminoethanol hydrochloride and thioglycollic acid on streptomycin and streptothricin has indicated that streptomycin is reversibly inactivated by cysteine and that the inactivation is not a property of the sulfhydryl group nor limited to cysteine (1). Brink *et al.* (2), in connection with structure studies, found that carbonyl group reagents such as hydroxylamine and semicarbazide cause complete inactivation. Donovan *et al.* (9), have extended these studies with the carbonyl reagents semicarbazide, thiosemicarbazide, hydroxylamine hydrochloride and hydrazine hydrate, confirming and amplifying previous observations on the reaction of these agents with streptomycin. More recently the inactivation of streptomycin by several reducing agents, some of which are included in this work, has been described by Bondi *et al.* (3).

Following these observations we have treated streptomycin in aqueous solutions with a large number of agents, both oxidizing and reducing, in an attempt to study inactivation in terms of such reactions.

## EXPERIMENTAL

Solutions of streptomycin hydrochloride were prepared from a dry material having a potency of 200–250  $\gamma$ /mg. and were admixed with solutions of the agents listed in Tables I and II. In each case the concentrations indicated in the tables are the final concentrations after mixture with the streptomycin solutions. After allowing to stand for 1–4 hours at room temperature, the solutions were submitted for assay by a modified agar dilution streak technique patterned after that described by Waksman and Reilly (4). Dilutions were made in increments of 10–30%, with the percentage increments decreasing as the dilutions increased. The minimal inhibiting dilution is defined as that dilution at which greater than 50% bacteriostasis of the test organism occurred.



TABLE I

Agent	Streptomycin Hydrochloride	Minimal Inhibiting Dilution		
		<i>E. coli</i>	<i>B. subtilis</i>	<i>S. aureus</i>
—	1 mg./ml.	340	>800	550
Potassium permanganate 0.025 <i>N</i>	—	<5	<5	<5
Potassium permanganate 0.025 <i>N</i>	1 mg./ml.	<5	<5	<5
—	1 mg./ml.	350	900	700
Potassium periodate 0.05 <i>M</i>	—	<5	<5	<5
Potassium periodate 0.05 <i>M</i>	1 mg./ml.	<5	<5	<5
—	1 mg./ml.	370	>800	500
Nitric acid 1.0 <i>N</i>	—	90	175	125
Nitric acid 1.0 <i>N</i>	1 mg./ml.	90	250	125
—	1 mg./ml.	420	>800	>800
Hydrogen peroxide 0.3%	—	80	325	250
Hydrogen peroxide 0.3%	1 mg./ml.	65	350	325
—	1 mg./ml.	440	>800	550
Sodium chlorate 0.5%	—	15	50	25
Sodium chlorate 0.5%	1 mg./ml.	7.5	30	12.5
—	1 mg./ml.	440	>800	600
Bromine <sup>1</sup>	—	<5	<5	<5
Bromine <sup>1</sup>	1 mg./ml.	440	>800	600
—	1 mg./ml.	400	>800	650
Potassium chlorate 1.0% <sup>2</sup>	—	<5	<5	<5
Potassium chlorate 1.0% <sup>2</sup>	1 mg./ml.	400	>800	650
—	1 mg./ml.	370	>800	650
Potassium chromate 5%	—	<5	<5	<5
Potassium chromate 5%	1 mg./ml.	350	>800	550
—	1 mg./ml.	380	>1000	630
Potassium bichromate 5% <sup>2</sup>	—	90	90	50
Potassium bichromate 5% <sup>2</sup>	1 mg./ml.	375	900	500

<sup>1</sup> Samples shaken with 1% Br<sub>2</sub> in CCl<sub>4</sub>. Excess Br<sub>2</sub> removed after 2 hours by shaking with cyclohexene.

<sup>2</sup> Conducted at pH 2.0.

TABLE II

Agent	Streptomycin H <sub>2</sub> dihydrochloride	Minimal Inhibiting Dilution		
		<i>E. coli</i>	<i>B. subtilis</i>	<i>S. aureus</i>
—	1 mg./ml.	250	> 500	450
Cysteine 2.0% <sup>1</sup>	—	15	15	15
Cysteine 2.0%	1 mg./ml.	225	> 500	375
—	1 mg./ml.	275	> 500	> 500
Hydroxylamine 0.01%	—	< 5	< 5	5
Hydroxylamine 0.01%	1 mg./ml.	90	250	180
—	1 mg./ml.	250	> 500	500
Sodium hypophosphite 40%	—	< 5	< 5	< 5
Sodium hypophosphite 40%	1 mg./ml.	7.5	7.5	7.5
—	1 mg./ml.	380	> 1000	600
Sodium bisulfite 10%	—	140	180	180
Sodium bisulfite 10%	1 mg./ml.	100	325	130
—	1 mg./ml.	250	> 500	> 500
Stannous chloride 5.0%	—	65	180	110
Stannous chloride 5.0%	1 mg./ml.	70	> 500	> 500
—	1 mg./ml.	250	> 500	475
Sodium hydrosulfite 10%	—	50	70	50
Sodium hydrosulfite 10%	1 mg./ml.	35	> 500	70
—	1 mg./ml.	240	> 500	375
Sodium thiosulfate 50%	—	< 5	< 5	< 5
Sodium thiosulfate 50%	1 mg./ml.	250	> 500	450

<sup>1</sup> Assayed after two hours' time. See Fig. 1.

A wide range of concentrations of each agent was employed but, for sake of brevity, only the lowest concentrations found effective are listed in the Tables. Since some of the agents were, in themselves, bacteriostatic in the concentrations employed, and since the minimal bacteriostatic dilution values for the streptomycin solution varied somewhat from day to day, there are included in the tables, as control values, the bacteriostatic dilutions of the streptomycin and the reagent. Thus, the minimal inhibiting dilution values listed for any one combination and the controls thereof were obtained under identical assay conditions.

### DISCUSSION

In Table I are listed the effects of 9 oxidizing agents. Potassium permanganate and potassium periodate were very effective in de-

stroying streptomycin and had little or no bacteriostatic effect in themselves under the conditions of the test in the concentrations employed. It should be pointed out that potassium permanganate, while bactericidal, is immediately reduced by constituents of the assay agar. Nitric acid also was effective in destroying streptomycin but bacteriostatic concentrations were necessary. Hydrogen peroxide and sodium hypochlorite had intermediate effects whereas the remaining reagents had no apparent effect.

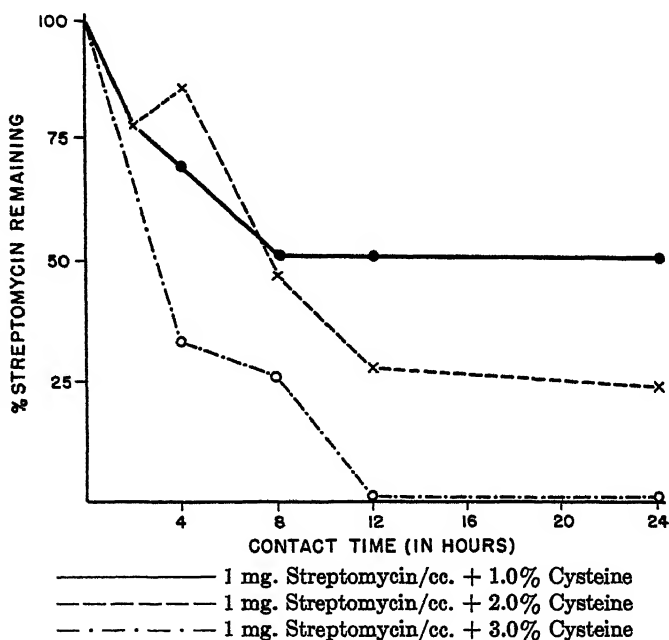


FIG. 1. Effect of Contact Time on the Inactivation of Streptomycin by Cysteine

The mechanism involved here is probably one involving oxidation of the streptomycin molecule by rather specific strong oxidants rather than the interference with an enzyme system. This is best exemplified by the effect of periodate, the action of which on polyhydroxy compounds is well known.

In Table II are listed the effects of 7 reducing agents. Included among these are cysteine and hydroxylamine which, it is recognized, may react with streptomycin through mechanisms other than reduc-

tion. Of the agents tried, sodium hypophosphite in high concentrations caused essentially complete inactivation. Sodium bisulfite, cysteine, hydroxylamine, stannous chloride and sodium hydrosulfite reduced the bacteriostatic action and changed the antibacterial spectra, while sodium thiosulfate had no effect. Of considerable interest is the change in antibacterial spectra which is best exemplified by sodium hydrosulfite. The addition of this substance, while having little differential bacteriostatic effect in itself, caused a marked drop of the activity against *Escherichia coli* and *Staphylococcus aureus* but had little effect on the activity against *Bacillus subtilis*. A possible explanation for this effect may lie in the reversal of interference with specific enzyme systems. The efficacy of sodium hypophosphite may be attributed to a combination of reductive action and inhibition of streptomycin by phosphates as described by Schatz and Waksman (5) and Woodruff and Foster (6, 7).

A factor in the inactivation of streptomycin by cysteine which has not been previously described is the time effect. Fig. 1 shows that several hours are necessary for the complete reaction to take place. The results would indicate that, in this case, the inactivation must proceed through reaction with the streptomycin molecule rather than through interference with an enzyme system concerned with antibiosis. Further study of this reaction, with regard to iodine reversal (1), after prolonged contact time, would seem desirable in clarification of this postulate.

#### ACKNOWLEDGMENT

We wish to acknowledge the assistance of the Bacteriology Control Department in conductance of the agar streak plate assays employed in these studies.

#### SUMMARY

1. The effects of a number of compounds, both oxidizing and reducing in nature, on the antibiotic activity of streptomycin have been described.

2. Inactivation of streptomycin may not logically be ascribed to an oxidizing nor reducing mechanism, but results from a specific reaction or an interference mechanism.

3. Potassium permanganate and potassium periodate are very specific in elimination of the antibiotic action of streptomycin. The efficacy and specificity of these agents suggests their use in develop-

ment of sterility tests or chemical assay procedures for streptomycin (8).

4. Several hours are required for the inactivation of streptomycin by cysteine. This fact is suggestive of a chemical reaction between cysteine and streptomycin, rather than a reversal of the mechanism of antibiosis by cysteine.

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# Inhibition of Creatine Phosphate and Acetylcholine Breakdown in Nerve Extracts by Procaine

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## INTRODUCTION

A new mechanism was recently proposed which can explain most of the phenomena associated with the propagation of a nerve impulse (1). The mechanism proposes, essentially, a competition between two enzyme systems for acetylcholine in the nerve. One of these enzymes is the well-known hydrolytic cholinesterase which yields choline and acetate while the second one causes a reaction between acetylcholine and creatine phosphate. The latter substance is presumed to be the source or energy of nerve impulse propagation (2).

Thimann (3) has suggested that nerve block anesthesia is the result of structural competition between acetylcholine and compounds of a similar structure. Among these structurally similar competitors is procaine and its relatives.

The work herein reported shows that: (a) an enzyme system is present in nerve extracts which catalyzes a reaction between acetylcholine and creatine phosphate, and (b) that this reaction is inhibited in the presence of procaine.

## EXPERIMENTAL

The enzyme preparation was made by homogenizing 1 g. of freshly excised frog sciatic nerves with 50 ml. of cold frog Ringer solution in a modified Waring Blendor. This mixture was used at once or stored at 5°C. for no longer than 1 hour.

The stock substrate upon which the tests were made consisted of standard frog Ringer solution which was made up to contain 0.2 mM/l. physostigmine salicylate, 1.0 mM/l. creatine phosphate (about 3.1 mg. P/100 ml.), and 1.0 mM/l. acetyl-

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choline bromide (about 23 mg./100 ml.). The solution was adjusted to pH 7.0 since acetylcholine hydrolyzes in basic solution while creatine phosphate hydrolyzes in acid solution.

The phosphocreatine was prepared as the calcium salt according to the directions of Lehniguer (in ref. 4) and converted to the sodium salt just before use by the addition of the calculated amount of sodium carbonate. The acetylcholine was obtained from the Eastman Kodak Co. and used without further purification.

Creatine phosphate was determined according to the method of Umbreit, Burris and Stauffer (4) and acetylcholine was estimated by means of the eserized frog rectus abdominis muscle preparation.

TABLE I

*A Reaction between Acetylcholine and Creatine Phosphate*

Enzyme extract	Before incubation		After 30 min. at 10°C.				
	Acetylcholine	Creatine phosphate	Acetylcholine left	Creatine phosphate left	Acetylcholine lost	Creatine phosphate lost	Ratio <sup>a</sup>
ml.	mM./l.*	mM./l.†	mM./l.	mM./l.	mM./l.	mM./l.	
2	1.1	none	1.0	—	0.1	—	
2	none	1.0	—	1.0	—	none	
2	1.1	1.10	0.1	0.15	1.0	0.95	1.05
2	2.2	1.10	1.2	0.05	1.0	1.05	0.95
2	1.0	2.10	0.2	0.98	0.8	1.12	0.73
2	2.1	2.00	0.5	0.60	1.6	1.50	1.07
1	2.0	2.10	0.9	1.10	1.0	1.10	0.97
2 (boiled)	1.10	1.10	1.0	1.05	0.1	0.05	

<sup>a</sup>  $\frac{\text{AcCh lost}}{\text{CrP lost}}$ .

\* Standard deviations were  $\pm 0.3$ .

† Standard deviations were  $\pm 0.20$ .

#### THE REACTION BETWEEN ACETYLCHOLINE AND CREATINE PHOSPHATE

In all of these experiments a total of 50 ml. of the stock substrate, which contained the enzyme preparation, eserine, acetylcholine and creatine phosphate in the quantities indicated in Table I, were used. Immediately after mixing, 6 ml. were withdrawn, 4 ml. of which were added to 1 ml. of 10% calcium chloride saturated with calcium hydroxide, pH 8.7, for creatine phosphate determination, and the re-

maining 2 ml. were added to 2 ml. of 0.1 N HCl and used for the estimation of acetyl choline.

In determining creatine phosphate, the color was developed as directed (4) and measured with a Coleman Universal Spectrophotometer. The creatine phosphate values were read from a curve made from standard determinations. It is assumed that creatine phosphate is represented in this technique by the apparent inorganic phosphate not precipitated by calcium ions.

TABLE II  
*The Inhibiting Effect of Procaine*

Enzyme extract	Before incubation				After 30 min. at 10°C.			
	Acetylcholine	Creatine phosphate	Procaine hydrochloride	"R"	Creatine phosphate left	Creatine phosphate lost	Inhibited (S)	$\frac{100-S}{R}$
ml.	mM./l.*	mM./l.†	mM./l.		mM./l.	mM./l.	per cent	
2	1.1	1.20	none	—	0.30	0.90	—	—
2	1.2	1.20	none	—	1.10	0.10	—	—
(boiled)								
2	1.1	1.00	1.0	52	0.30	0.70	22	1.5
2	1.0	1.30	2.0	33	0.86	0.44	51	1.1
2	1.1	1.10	3.0	25	0.78	0.32	65	1.4
2	1.0	1.05	4.0	20	0.80	0.25	72	1.4
2	1.2	1.12	10.8	10	0.99	0.13	85	1.5
2	1.0	5.20	10.0	10	5.09	0.11	87	1.3

$$"R" = \frac{\text{AcCh}}{\text{CrP} + \text{AcCh}} \times 100.$$

\* Standard deviations were  $\pm 0.3$ .

† Standard deviations were  $\pm 0.25$ .

For the acetylcholine estimations the samples were neutralized and an aliquot used to cause the frog rectus to contract. This contraction was then approximately matched with a standard amount of acetylcholine and the actual amount of substance in the test sample calculated. The standard error was  $\pm 3\%$ .

The results of these experiments are summarized in Table I. Each value represents the average of 10 trials.



## THE INHIBITING EFFECT OF PROCAINE

These experiments were made as in part I except that various amounts of procaine hydrochloride were added to the substrate. Only the creatine phosphate values are reported since the presence of procaine makes the determination of acetylcholine with the rectus abdominis muscle preparation impossible. This phenomenon is being investigated quantitatively.

The results of these experiments are shown in Table II. Each value represents the average of 10 trials.

## DISCUSSION

It has been shown that there is an enzyme system in frog sciatic nerve extract which catalyzes a reaction between acetylcholine and creatine phosphate. This reaction appears to be a mole-for-mole one. It appears probable that this reaction occurs when a nerve impulse travels along a nerve since it is known that stimulating a nerve causes a release of acetylcholine. The appearance of acetylcholine at the point of stimulation may also explain the electrical phenomenon since it has been shown by Beutner and Barnes (5) that acetylcholine gives a negative potential at the phase boundary between cholesterol and saline.

The reaction between acetylcholine and creatine phosphate is inhibited by procaine and this inhibition seems to be due to a competition between acetylcholine and procaine for the same enzyme receptors. This is shown by the data as a fairly constant relationship exists between the degree of inhibition and the mole ratio of procaine to procaine plus acetylcholine.

These findings point to the mode of action of procaine as a nerve block anesthetic. It seems to prevent the spread of the nerve impulse by preventing the release of energy by blocking the apparently essential reaction between acetylcholine and the energy-rich creatine phosphate.

The apparent failure of an exactly mole-for-mole competition may be explained by noting that the procaine *ion* seems to be the active form for anesthesia (6). At the pH of the experiments, only a portion of the procaine molecules are immediately present as procaine ions, the rest being in the free-base form. Further work is now in progress in this laboratory.

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# An Improved Method for the Preparation of Coenzyme I

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## INTRODUCTION

In our opinion there exists no simple and satisfactory method for the preparation of coenzyme I. It is noteworthy that this substance, otherwise known as cozymase, codehydrase I and diphosphopyridine nucleotide, should be so difficult to purify. Practically all purification methods published thus far follow the same general procedure and the more recent methods, with the exception of one step in Jandorf's method (1), do not appear to have added anything especially valuable.

While the majority of workers have employed yeast as the source of coenzyme I, Ochoa (2) has suggested that fresh canine muscle is an equally good source. Warburg and Christian (3) claim that horse erythrocytes constitute the best source for coenzyme I, coenzyme II and adenine nucleotide.

The method most frequently used consists in extracting yeast with water at 80–85°C., after which neutral lead acetate is added to precipitate impurities. The excess of lead is usually removed either by treatment with hydrogen sulfide or potassium oxalate. After this, the coenzyme I is precipitated by mercuric nitrate, phosphotungstic acid, silver nitrate or cuprous chloride. Shaking with dilute sulfuric acid, amyl alcohol and ether is employed to decompose the phosphotungstic acid precipitate, and saturation with hydrogen sulfide is used to decompose the other precipitates. The coenzyme is finally precipitated by adding alcohol to a concentration of 90% and may be purified further by the use of adsorbents and by other procedures. In the method of von Euler, Albers and Schlenk (4) all of the precipitations mentioned above are used. In our hands these precipitations required one week to carry out. We can confirm the statement made by these authors that precipitation of the coenzyme is not always complete and that a part of it is likely to be destroyed during purification. We made coenzyme I by the method of von Euler *et al.*, carried through the stage of alcohol precipitation, which contained 6.8% of phosphorus. At this

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\* Government of India Scholar.

point we wish to call attention to the fact that the phosphorus content of coenzyme I cannot always be used as a criterion of purity, since we have prepared one sample by a modification of our method which, while 30% more active than material we prepared by the method of von Euler *et al.*, contained only 4% of phosphorus; while our most active coenzyme I preparations, containing 8.9% of phosphorus, are about 12-fold more active. As will be shown later, the phosphorus content of *purified* coenzyme I which has been doubled in activity by two adsorptions upon Norit is only slightly higher than material of half this activity. It should be noted here that we are not assuming that our coenzyme preparations, made by the method of von Euler *et al.*, are of the same activity as their own.

Warburg and Christian (3) used horse erythrocytes which were washed and laked, after which protein was removed by adding acetone. From the acetone extract coenzyme I, coenzyme II and adenine nucleotide were precipitated by mercuric acetate. The precipitate was decomposed by hydrogen sulfide and the solids precipitated by acetone. Adenine nucleotide was removed as the barium salt, insoluble in water. Coenzyme II was removed as the barium salt insoluble in alcohol, leaving behind coenzyme I, which was precipitated with mercuric acetate. The precipitate was decomposed with hydrogen sulfide and the coenzyme I was precipitated with acetone. Further purification was achieved by dissolving the material in anhydrous 0.1 *N* hydrochloric acid in methanol and precipitating with ethyl acetate. Coenzyme I was next precipitated by lead, the precipitate was decomposed by hydrogen sulfide and precipitated from aqueous solution by acetone. The product was contaminated by a fluorescent substance, which could be removed by oxidation with bromine.

Ohlmeyer (5) extracted Lowenbrau yeast with hot water, removed impurities with lead acetate, and precipitated the coenzyme with silver nitrate. The silver complex was decomposed with hydrochloric acid. It was then precipitated as the phosphotungstate and this precipitate was decomposed with acetone. Further purification was achieved by fractional precipitation with lead acetate, with silver nitrate, with phosphotungstic acid, then with cuprous chloride and once again with silver nitrate. Ohlmeyer's method, with minor modifications, was used by Ochoa (2) for the purification of coenzyme I from dog muscle.

Williamson and Green (6), starting with extract made at 85°C. from yeast, precipitated impurities with lead acetate and precipitated the coenzyme with silver nitrate. The phosphorus content of their alcohol-precipitated product had a value of 6.56%. The authors concluded on this basis, as well as from the nicotinic acid content and spectroscopic analysis, that their product was about 65% pure.

In 1941 Jandorf (1) added the technique of charcoal adsorption for the purification of coenzyme I to the cuprous chloride precipitation. Charcoal had been used first by Green, Needham and Dewan (7) to remove traces of coenzyme I from enzyme solutions. His procedure consisted in making an extract of brewer's yeast with hot acid, removal of impurities with lead acetate, adsorption of the coenzyme on Norit, elution of the coenzyme by shaking with water and amyl alcohol for many hours and precipitation with cuprous chloride. The final precipitation was by alcohol. The product showed no fluorescence and contained the theoretical amount of phosphorus. Its biological potency was claimed to be the same as that of a "pure" specimen obtained from Warburg's laboratory.

It appears to us that one of the chief defects of methods which start with yeast is the extraction, together with the coenzyme, of a large amount of yeast gum, a substance which interferes with all later procedures. We have succeeded largely in avoiding this by extracting moist baker's yeast at room temperature with a mixture of ether, alcohol and sulfuric acid. The mixture is then centrifuged and the clear, brown solution is decanted. The coenzyme I is next precipitated by adding 2 volumes of 95% alcohol, followed by cooling overnight. The coenzyme is centrifuged down, washed with absolute alcohol and absolute ether and dried. The dry material is an almost white powder which dissolves instantly in water to give a nearly clear solution. It is from 10 to 20-fold more active than the moist yeast from which it is made. The material gives no biuret or ninhydrin tests. The percentage of phosphorus is between 0.4 and 0.6. The ash amounts to 37%. Spectroscopic analysis shows much potassium, a moderate quantity of magnesium, small amounts of phosphorus, copper and silicon, and traces of sodium and calcium.

Further purification of the coenzyme can be achieved by adsorption upon acid-washed Norit. The quantity of Norit required is no greater than the weight of crude coenzyme used and we find that only a few minutes are needed for complete adsorption. The adsorption complex is centrifuged, washed with distilled water and then with dilute ammonia, whereby a considerable amount of impurity is removed. After again washing with water, the coenzyme is eluted by shaking with water and amyl alcohol, centrifuged, filtered, evaporated to small volume, clarified by mixing with 2 volumes of absolute alcohol and centrifuging, precipitated by adding excess of alcohol and a drop or two of sulfuric acid and centrifuging, washed with absolute alcohol and with absolute ether and dried *in vacuo*.

Our once-adsorbed coenzyme I is about 1000-fold more active than the moist yeast (71% water) from which it is prepared, or 290-fold more active than dry yeast. We have made comparative determinations of coenzyme I activity by using purified lactic apodehydrogenase, purified diaphorase, lactate, hydrocyanic acid and methylene blue in a stream of oxygen-free nitrogen. We have made other determinations by using dried and then washed yeast, phosphate buffer, fructose-1,6-diphosphate and methylene blue. Here the dehydrogenase acts upon diphosphoglyceric aldehyde. While we believe that by far the most significant property of coenzyme I is its ability to function as a catalyst,

it appeared of interest to analyze it for its elements and also to chart its absorption in the ultraviolet. The phosphorus content of once-adsorbed coenzyme I varies from 8.5 to 8.9%. Nitrogen by the micro-Dumas was 11.64 and 11.61% and hydrogen 6.86, 5.66 and 6.03%. Carbon was 31.95, 31.85 and 32.03%. The preparation analyzed here for nitrogen, hydrogen and carbon had been dried at room temperature *in vacuo* over phosphorus pentoxide. Upon keeping overnight at 50°C. in a vacuum over phosphorus pentoxide the weight decreased by 1.5%.

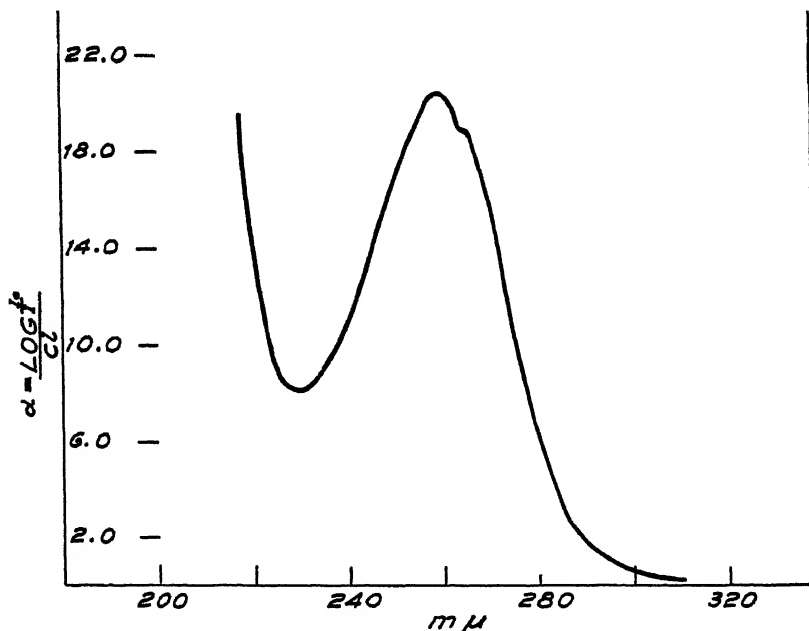


FIG. 1.—Adsorption Spectrum of Once-Adsorbed Coenzyme I

The absorption spectrum for a once-adsorbed coenzyme I sample is shown in Fig. 1. Here we employed a concentration of 0.05 mg./cc. in a 1 cm. cell and used a hydrogen discharge tube with a Beckman spectrophotometer. The spectrum is very similar to the spectra of coenzyme I obtained by von Euler *et al.* (4) and by others. However, we do not think that too much weight can be placed upon the spectrum of coenzyme I preparations, inasmuch as inactivated material may very well interfere.

We have considered it of greatest importance to adsorb our purified coenzyme I for a second time upon acid-washed Norit to observe whether any further purification could be achieved and have been surprised to obtain a product which, after drying at 50°C. *in vacuo* over phosphorus pentoxide, is 100% more active than our most active material by one adsorption. The phosphorus content of this preparation is 8.9%; nitrogen 11.35, 10.53, 10.44, 11.35%; hydrogen 5.76 and 5.71%, while carbon is 32.83 and 33.14%. These figures are not far different from those for once-adsorbed coenzyme I and they seem to show that the once-adsorbed coenzyme is contaminated by some inactive compound which has practically the same composition as the twice-adsorbed material. However, our analyses for the twice-adsorbed material do not agree with values calculated for the generally accepted formula for coenzyme I, which are: phosphorus 9.3, nitrogen 14.7, hydrogen 4.06 and carbon 37.9%.

TABLE I

Operation number	Material	Pure coenzyme I calculated present	Actual yield	Yield	Fold purified
—	1 kg. moist baker's yeast	mg. 500	mg. —	per cent —	0
1	Crude coenzyme	60-120	7000	12-24	12-14
2	Once-adsorbed coenzyme	40-80	80-160	4-8	1000
3	Twice-adsorbed coenzyme	5-10	5-10	1-2	2000

Table I shows how the concentration of coenzyme I increases during 3 operations. For the sake of comparison we have assumed our twice-adsorbed coenzyme to be 100% pure, but we do not believe this to be true.

### THE METHOD

Mix 1 pound (454 gm.) of compressed, starch-free baker's yeast with 350 cc. of ether. Add 22 cc. of concentrated sulfuric acid to 350 cc. of 95% alcohol cautiously and cool to room temperature. Add this acidified alcohol to the yeast and ether and stir with a wooden stick for about 15 minutes, when the mixture should be uniform. Centrifuge for 5 or 10 minutes and decant the clear, brown solution of coenzyme I into a large Erlenmeyer flask. When all of the mixture has been centrifuged, precipitate the coenzyme by adding 2 volumes of 95% alcohol, mixing and chilling in the



ice chest overnight or for a longer period. Decant as much as possible of the nearly clear supernatant and centrifuge the rest. Stir the precipitate with excess of absolute alcohol, centrifuge and discard the supernatant. Repeat this washing with absolute alcohol. Now wash in the same manner with absolute ether. After discarding the ether supernatant dry the crude coenzyme I *in vacuo* over drierite or fused calcium chloride. The yield will be about 2 g. It is practicable, although not ordinarily desirable, to obtain still more coenzyme I by reextracting the yeast residue with 360 cc. of 95% alcohol, 240 cc. of water and 15 cc. of concentrated sulfuric acid. The mixture is stirred mechanically for about 1 hour and filtered in the cold overnight on fluted filter papers. The next day the clear, yellow filtrate is mixed with 2.5 volumes of 95% alcohol and the precipitate treated as usual. When dry this second yield will amount to about 1.2 g. If large quantities of coenzyme I are desired, one can start with 10 lbs. of baker's yeast and mix this with the ether and acidified alcohol in a large enameled pail. The supernatant solution obtained upon centrifuging is precipitated by the 95% alcohol in a 20-liter carboy.

### FURTHER PURIFICATION

To prepare coenzyme I of a high degree of purity dissolve 10 g. of the crude powder in 1 l. of distilled water and stir for 4 or 5 minutes with 10 g. of acid-washed Norit. (We have used Pfanstiehl type A Norit which we have soaked in concentrated hydrochloric acid, washed *ad nauseam* with water, then with dilute ammonia, then with water. After drying the pH of a 1% suspension of the product was 3.6.) The coenzyme-Norit complex is centrifuged down and washed twice by stirring with about 1 l. of distilled water and then centrifuging. It is then stirred up with dilute ammonia (0.5 cc. of 28% ammonia to 500 cc. of water) and then centrifuged. This treatment with ammonia is repeated. The coenzyme-Norit complex is then washed twice with water, using about 500 cc. each time. The complex is now suspended in about 100 cc. of water and 10 cc. of isoamyl alcohol are added. The mixture is shaken in a bottle for about 1 hour, after which it is centrifuged. After pouring off the supernatant, a second elution is made by mixing the Norit precipitate with a second 100 cc. of water and 10 cc. of isoamyl alcohol and shaking for one hour. After centrifuging, this supernatant is added to the first supernatant. The combined supernatants, containing some charcoal, are filtered and refiltered through a wet filter paper and the filtrate, after making slightly alkaline with ammonia to prevent hydrolysis, is evaporated at a temperature of not more than 40°C. by placing it in a clean porcelain evaporating dish on a steam bath and cooling with an electric fan. When the volume of the coenzyme solution is only 3 or 4 cc. the evaporating dish is wiped on the bottom and the solution is carefully poured into a small graduate to measure its volume. It is then poured into a 50 cc. centrifuge tube. Two volumes of absolute alcohol are added and, after stirring, the preparation is centrifuged clear. This treatment gets rid of certain impurities, among which are traces of charcoal. If this does not remove the charcoal more alcohol can be added and the preparation can be centrifuged again. The supernatant is poured into a second 50 cc. centrifuge tube and mixed well with about 40 cc. of absolute alcohol. About 2 drops of sulfuric acid, 7.5 N, are added with stirring and the coenzyme I, which separates as tiny spheroids, is centrifuged down. If the coenzyme shows a tendency to remain in colloidal suspension

more sulfuric acid must be added. The clear supernatant is discarded and the precipitate is stirred up with about 40 cc. of absolute alcohol and centrifuged again. This washing with absolute alcohol is repeated twice. The preparation is then washed with absolute ether. After discarding the ether supernatant the preparation is dried *in vacuo* over phosphorus pentoxide. The yield from 10 g. of crude material will be 0.12 to 0.20 g.

### THE SECOND ADSORPTION

Dissolve 200 mg. of the purified (once adsorbed and eluted) material in 50 cc. of water, add 1 g. of acid-washed Norit and mix for 4 or 5 minutes. Centrifuge the Norit-coenzyme I complex and wash once by stirring with 80 cc. of distilled water and centrifuging. Wash once by stirring with 80 cc. of water and 0.4 cc. of 28% ammonia and centrifuging. Wash once by stirring with 80 cc. of water and centrifuging. The material is now shaken for 40 minutes with 80 cc. of water and 5 cc. of amyl alcohol. The preparation is centrifuged and filtered through a wet filter paper. The filtrate is made alkaline with a drop of 28% ammonia and evaporated at 35°C. to about 1 cc. in a 10 cm. evaporating dish on a steam bath by means of an electric fan. The dish is wiped on the bottom and the coenzyme solution is transferred to a 50 cc. centrifuge tube, using 4 cc. of absolute alcohol. After mixing, the turbid solution is centrifuged. The clear supernatant is now mixed with 5 cc. more of absolute alcohol and 1 drop of 7.5 *N* sulfuric acid is added. The coenzyme is centrifuged down, washed twice with absolute alcohol, once with absolute ether and then dried *in vacuo* over phosphorus pentoxide. The yield from 100 mg. of material will be only 15 to 30 mg.

It should be added that during centrifuging we have kept our 50 cc. centrifuge tubes covered with paper caps to prevent evaporation or entrance of foreign matter.

### ACKNOWLEDGMENT

We wish to express our gratitude to the Rockefeller Foundation for financial aid and to Dr. L. I. Diuguid for running determinations for nitrogen, carbon and hydrogen.

### SUMMARY

A method is described for preparing crude coenzyme I in the form of a white powder containing 0.4–0.6% of phosphorus. An adsorption procedure is described for preparing from this crude material a product containing 8.5 to 8.8% of phosphorus which is nearly 1000-fold more active than the moist yeast used as raw material, or 290-fold more active than dry yeast. By means of a second adsorption a product is obtained which is about 2000-fold more active than the moist yeast and which contains about the same percentage of phosphorus, nitrogen, carbon and hydrogen as the product obtained by one adsorption. These figures do not agree with those required for the accepted formula for coenzyme I.

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# A Method for Determining the Avidin-Combinability of Biotin Analogs<sup>1</sup>

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## INTRODUCTION

The ability of avidin to combine with a number of biotin analogs has been demonstrated (1, 2, 3). Compounds having activity in promoting growth of yeast have been considered to combine with avidin if growth was negated by avidin. Derivatives having no growth-promoting activity for yeast have been considered to combine with avidin if such derivatives could displace biotin from the avidin-biotin combination. du Vigneaud and coworkers have concluded that the cyclic urea ring is essential for avidin-combinability but that the remaining structure of the molecule is a factor in modifying the affinity of avidin for biotin analogs (4).

Previous experiments concerning the combination of biotin and its analogs with avidin have been limited largely to those of a qualitative nature and to the use of yeast as an assay organism. This paper describes procedures found useful for determining quantitatively the *relative affinity* of avidin for biotin analogs rather than the ability of such analogs to *displace* biotin from combination. In measuring the "relative affinity" of biotin and an analog of biotin for avidin, biotin and avidin are used in stoichiometric amounts, while the amount of analog present is varied. The "relative affinity" for avidin is then expressed arbitrarily as the ratio of the concentration of analog to biotin at which one-half of the biotin remains free and available for growth of the test organism. It can be assumed that at this point, one-half of the avidin is combined with biotin, the other 50% has combined with

<sup>1</sup> A preliminary report appeared in the *Federation Proc.* 5, 160 (1946).

the analog. It is obvious that the ratio will be low for analogs having high affinity for avidin and higher for those analogs which do not combine readily with avidin. Because of the firmness of the avidin combination, affinity measurements have been found, in the present study, to be the more sensitive method of determining avidin-combinability. In addition, the amount of material required for a determination of avidin-combinability by affinity measurements is much less than that required for the displacement procedure.

### EXPERIMENTAL

The methods employed in this study were those commonly used in microbiological assays with lactic acid bacteria. Biotin was determined microbiologically with *Lactobacillus arabinosus* (5). The extent of bacterial growth was determined either turbidimetrically after 24 hours of growth or by titration of the acid produced after 72 hours of growth.

Avidin was used in the form of a concentrate containing 50 units of activity/g. Sterilization of avidin solutions was effected by filtration through a fritted glass filter. Combinability studies were carried out directly in the microbiological assay medium prior to seeding with *L. arabinosus*.

In the determination of avidin-combinability the response of *L. arabinosus* to biotin is first determined. Secondly, the activity of the avidin preparation in combining with biotin is measured by the relative growth of *L. arabinosus* following the aseptic addition of varying amounts of avidin to previously sterilized tubes containing a constant amount of biotin. Lastly, the relative affinity of avidin for biotin and biotin analog is determined by relative growth of *L. arabinosus* following the addition of avidin to mixtures of biotin and analog. The standard curve is used to determine the amount of biotin uncombined with avidin and, thus, indirectly the amount of analog taking part in the reaction. The data are readily obtainable in a single microbiological assay after the approximate activity of the avidin preparation is known. Appropriate calculations are made if biotin and avidin are not used in exactly equivalent amounts. Suitable corrections must also be applied if the analog under investigation has some activity in promoting growth of *L. arabinosus*.

That utilization of available biotin by the organism during growth, thus changing the ratio of analog to biotin, fails to influence the affinity ratios obtained was demonstrated with desthiobiotin as an example. Because of the firmness with which biotin or analog combines with avidin the affinity ratio does not change appreciably during growth of *L. arabinosus* and the ratios obtained are independent of the period of growth. The affinity ratio is independent of the amount of biotin and avidin used, provided the two are used in approximately equivalent amounts.

### RESULTS AND DISCUSSION

The method is illustrated by a study of the avidin-combinabilities of the unnatural enantiomorph of biotin and the related racemates *dl*-

allobiotin and *dl*-epiallobiotin (Fig. 1). These compounds were kindly furnished by Merck and Co. through the cooperation of Dr. Karl Folkers and Dr. Walther Ott. The epibiotins have never been identified and apparently do not exist (6). *l*-Biotin, *dl*-allobiotin, and *dl*-epiallobiotin are inactive in promoting growth of *L. arabinosus* (7, 8). The data of Table I illustrate the results obtained and indicate the type of data essential for determining the avidin-combinability of other biotin analogs.

Avidin had no significant affinity for *l*-biotin or *dl*-allobiotin but showed some affinity for *dl*-epiallobiotin. An affinity ratio of approxi-

TABLE I

*Data for Determining the Relative Affinity of Avidin for Biotin, l-Biotin, dl-Allobiotin and dl-Epiallobiotin*

Tube No.	Components					Relative turbidity
1	0	$\gamma$ biotin				96
2	0.00025	biotin				168
3	0.00050	biotin				216
4	0.00075	biotin				276
5	0.00100	biotin				320
6	0.00150	biotin				405
7	0.00250	biotin				530
8	0.00250	$\gamma$ biotin +	20	$\gamma$ avidin		485
9	0.00250	biotin	50	avidin		395
10	0.00250	biotin	100	avidin		240
11	0.00250	biotin	200	avidin		95
12	0.00250	$\gamma$ biotin +	0.002	$\gamma$ <i>l</i> -biotin +	100 $\gamma$ avidin	236
13	0.00250	biotin	0.005	<i>l</i> -biotin	100 avidin	240
14	0.00250	biotin	0.010	<i>l</i> -biotin	100 avidin	234
15	0.00250	biotin	0.020	<i>l</i> -biotin	100 avidin	240
16	0.00250	$\gamma$ biotin +	0.002	$\gamma$ <i>dl</i> -allobiotin +	100 $\gamma$ avidin	240
17	0.00250	biotin	0.005	<i>dl</i> -allobiotin	100 avidin	240
18	0.00250	biotin	0.010	<i>dl</i> -allobiotin	100 avidin	250
19	0.00250	biotin	0.020	<i>dl</i> -allobiotin	100 avidin	262
20	0.00250	$\gamma$ biotin +	0.002	$\gamma$ <i>dl</i> -epiallobiotin +	100 $\gamma$ avidin	305
21	0.00250	biotin	0.005	<i>dl</i> -epiallobiotin	100 avidin	390
22	0.00250	biotin	0.010	<i>dl</i> -epiallobiotin	100 avidin	440
23	0.00250	biotin	0.020	<i>dl</i> -epiallobiotin	100 avidin	485

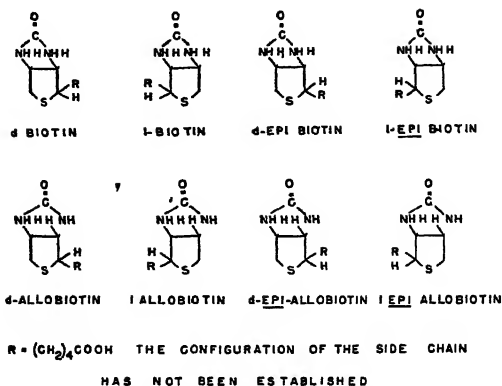
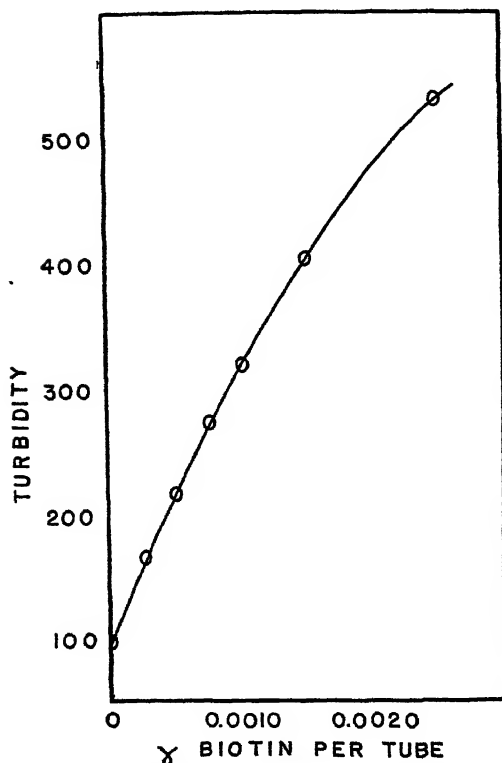


FIG. 1. The Spatial Isomers of Biotin

FIG. 2. The Response of *Lactobacillus arabinosus* to Biotin

mately 6 for *dl*-epiallobiotin may be calculated from these data (see Figs. 2 and 3 and Table II). *dl*-Allobiotin and *dl*-epiallobiotin solutions were not subjected to heat because of the instability of the *trans* urea ring. Because of the instability of these compounds avidin-combinability determinations were carried out directly following preparation of the solutions.

Fig. 1 may be helpful in following the discussion concerning the spatial configuration essential for avidin-combinability. It should be

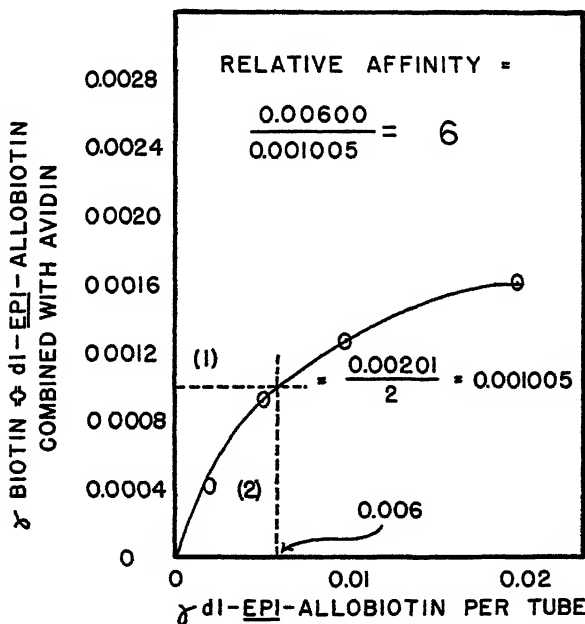


FIG. 3. The Relative Affinity of Avidin for Biotin and *dl*-Epi-allobiotin. (1) is the point at which one-half of the avidin is combined with biotin and one-half combined with analog; (2) is the level of analog required to attain this condition.

pointed out that the studies of Harris *et al.* (6) have demonstrated that the members of the biotin series of compounds possess a *cis* urea ring while the members of the allobiotin series possess a *trans* urea ring. *dl*-Epibiotin, therefore, differs from *dl*-biotin in being epimeric with it at position 2, and *dl*-epiallobiotin differs from *dl*-allobiotin likewise in being epimeric at position 2. It should be emphasized that the configuration on position 2 is unknown with respect to the relationship between



TABLE II

*Calculation of the Relative Affinity of Avidin for Biotin and dl-Epiallobiotin (Data of Table I)*

The activity of the avidin preparation in combining with biotin:

(1) Turbidity (tubes 8-11)	(2) Biotin added $\gamma$	(3) Biotin un- combined (Fig. 2) $\gamma$	(4) Biotin com- bined (2-3) $\gamma$	(5) Avidin prepa- ration used $\gamma$	(6) Biotin com- bined per 100 $\gamma$ avidin preparation. $\gamma$
485	0.00250	0.00210	0.00040	20	0.00200
395	0.00250	0.00145	0.00106	50	0.00212
240	0.00250	0.00060	0.00190	100	0.00190
				Average	0.00201

The relative amounts of biotin and *dl*-epiallobiotin combining with the avidin preparation:

(7) Turbidity (tubes 20-23)	(8) <i>dl</i> -Epiallo- biotin added $\gamma$	(9) Biotin added $\gamma$	(10) Biotin un- combined (Fig. 2) $\gamma$	(11) Biotin used in excess of avidin equiv- alence (0.00250- 0.00201) $\gamma$	(12) Biotin equiva- lent to <i>dl</i> - epiallobiotin combined with avidin (10-11) $\gamma$
305	0.002	0.00250	0.00092	0.00049	0.00043
390	0.005	0.00250	0.00140	0.00049	0.00091
440	0.010	0.00250	0.00175	0.00049	0.00126
485	0.020	0.00250	0.00210	0.00049	0.00161

A curve is constructed (Fig. 3) in which the values of column 12 are plotted against the corresponding values of column 8. The relative affinity is then obtained from the curve.

side chain and nearest N and that the nomenclature applied in the biotin series does not necessarily bear any relationship to that assigned in the allobiotin series.

The relative affinities found for the various isomers of biotin permit the conclusion that for maximal avidin-combinability a *cis* urea ring must exist in the molecule. Some degree of avidin-combinability is possible, however, with a *trans* urea ring provided the relationship between side chain and one nitrogen atom of the urea ring bear the same relationship to each other as that existing in *d*-biotin. It is im-

possible with the present data to designate which of the nitrogen atoms is involved.

The inactivity of *l*-biotin in combining with avidin offers a possible means of resolving the synthetic racemate since avidin-*d*-biotin is capable of ready separation from unreacted *l*-biotin. *d*-Biotin is obtainable readily from the avidin-biotin combination.

### SUMMARY

A procedure for determining the relative affinity of avidin for biotin analogs possessing little or no microbiological activity *per se* has been presented. The method has been illustrated by a determination of the avidin-combinability of the available spatial isomers of biotin.

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# The Effect of Ultraviolet Light on Cotton Cellulose and Its Influence on Subsequent Degradation by Microorganisms <sup>1</sup>

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## INTRODUCTION

One of the problems which received a great deal of recognition during the war was the deterioration of cotton textiles in tropical theaters of war. Many industrial, governmental, and private laboratories were active in the study of microbiological decay of textiles by investigating such aspects as the application of antimicrobial agents to fabrics, the prevention of the decay through an altering of the chemical nature of the cotton fiber itself, and the more fundamental aspects of the problem involving the isolation and identification of the causal microbiological agents and the investigation of the biochemical mechanism(s) of the cellulolytic process.

Since it is known that exposure of cotton fabric to sunlight adversely affects the tensile strength, and since, in most cases in the field, fabric exposed to microbiological attack will also be subject to the influence of sunlight and other variable factors of the environment, this work was established to investigate the effect of light, temperature, humidity, and oxygen concentration on cotton cellulose and the subsequent degradation of that cellulose by microorganisms. Kaufman (1926) reported that the spectral region most effective in causing a deterioration of cellulose lies below 350 m $\mu$ , and Ellis, Wells and Heyroth (1941) and Launer and Wilson (1943) present further support for this contention. The roles that oxygen concentration and moisture play in this degradation, however, are still in controversy and, to our knowledge, there is

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<sup>1</sup> Work supported in part by funds from the U. S. Quartermaster Corps.

no evidence in the literature to indicate the effect of ultraviolet light on fungal decay of cellulose.

The working hypothesis upon which the research was predicated was that ultraviolet light causes a photochemical reaction to occur in cotton cellulose, and a modification of the cellulose will present an altered substrate to cellulose-decomposing fungi; consequently, biological degradation of cotton fibers will be altered by ultraviolet irradiation.

The prosecution of the work required a method for the uniform exposure of cotton cellulose to light under controlled temperatures, light intensities, oxygen tensions and humidities. Furthermore, the cellulose should be in such a form as to ensure a convenient and accurate method for subsequent testing for attack by microorganisms. For these reasons, the use of cotton fibers, which can be irradiated uniformly and under controlled conditions, and which can easily be subjected to microbiological attack, was adopted. Inasmuch as the index of degradation of immediate practical interest was the tensile strength, the Pressley technique was chosen, in which a bundle of cotton fibers, cut to standard lengths, was broken and the tensile strength per unit weight of bundle determined.

## MATERIALS AND METHODS

The cotton fibers to be treated were combed parallel and the ends cemented with beeswax in a layer one fiber thick across the arms of U-shaped frames. The frames were approximately 2.5 cm. across the arms and made of No. 26 B. and S. nichrome wire which possessed enough resiliency to keep the fibers stretched taut when properly mounted. Three to four mg. of cotton were used for each frame.

The frames holding the cotton were hung on the holder shown in two views in Fig. 1. Three rows of 6 frames each were hung on the 3 wire supports S of the holder H. The top wooden portion of the holder fitted tightly into the opening in the metal plate at the top of the chamber and through a rubber gasket R made a gas-tight seal. The fibers were exposed to the ultraviolet radiation from 2 lamps placed as shown in Fig. 1. Under these conditions two 3 mm. quartz plates Q and a 2.5 cm. layer of distilled water intervened between the fibers and each lamp. The lamps were so arranged that the quartz burners were at a distance of 25 cm. perpendicular to the plane of the fibers. The quartz plates were 15 cm. on a side and so permitted an exposure area of approximately 225 cm<sup>2</sup>.

Since it was necessary to maintain the fibers at a constant temperature during the period of exposure, the stainless steel frame of the treatment chamber TC was insulated on three sides with glass wool, and water at constant temperature circulated between the quartz plates on either side of the chamber as shown in the figure.

Distilled water was stored in the receptacle E immersed in the water bath where it was kept at the temperature of the bath, and was pumped from there through the chamber by the centrifugal pump D. The water was pumped through the copper coil F which served as a heat exchanger to assure equilibrium with the bath temperature before it was forced through the chamber.

Since the lamps gave off a considerable amount of heat, it was not possible to keep the treatment chamber temperature (as determined by the thermometer G in Fig. 1) the same as that of the water bath. However, a constant differential could be maintained between the bath and chamber; thus, when the bath was kept at a temperature of 35°C., a temperature of  $40^{\circ} \pm 1^{\circ}\text{C.}$  could be maintained in the treatment chamber; and when the bath was at 0°C., the chamber could be maintained at 15°C. These were the only two temperature conditions used in the experiments reported here. The water bath was maintained at the chosen temperature by heating and/or cooling coils depending on the room temperature.

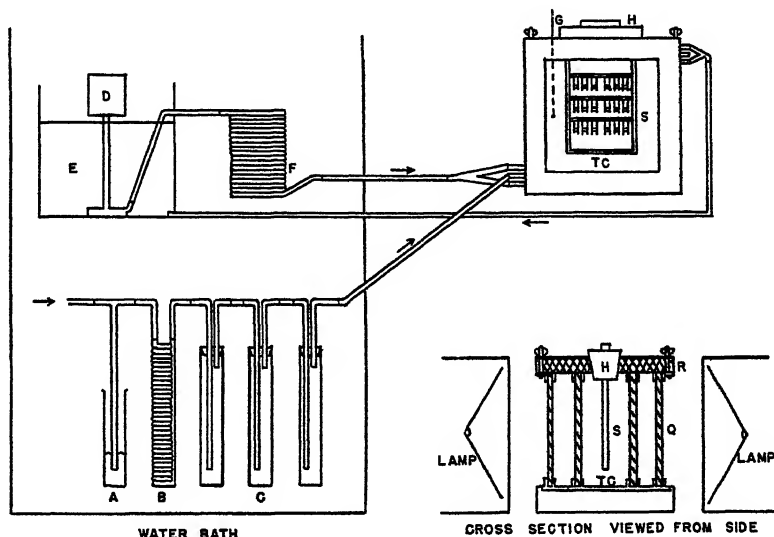


Fig. 1. Diagram of Apparatus Showing Treatment Chamber, Temperature and Humidity Control and Light Sources

The oxygen concentration in the chamber was controlled by using oxygen, air and nitrogen to give oxygen concentrations of 100, 21 and 0%, respectively. The gases were sent successively over the mercury valve A, through the cooling or warming coil B and the three humidity control towers labeled C. Glass beads were present in the towers to increase the gas-liquid contact area. Maintaining the temperature of the gas equal to that of the chamber was necessary as a precaution against condensation on the quartz windows.

Three different relative humidities were maintained in the treatment chamber by passing the gas through distilled water, saturated aqueous sodium chloride in towers C in Fig. 1, or through four towers of anhydrous barium chlorate (not illustrated), respectively. When the gas is at the temperature of the bath, it will have a relative humidity of 100% over distilled water, of approximately 76% over saturated aqueous sodium chloride and nearly 0% over barium chlorate. However, if the bath is at 35°C.

and the chamber at 40°C., the relative humidity in the chamber in the first case will be approximately 80% and, in the second, approximately 55% with no change in the case of the barium chlorate towers. These values were calculated from the data on the vapor pressures of saturated aqueous sodium chloride solutions and pure water at various temperatures. It should be noted that the relative humidity over a saturated solution of sodium chloride remains practically constant over a range of temperature from 0° to 40°C., varying only from 75 to 78%.

Two Hanovia SC 5010 analytical model quartz mercury vapor lamps were used as the light sources. The burners in these lamps are rated to give the high intensity band spectrum characteristic of the high pressure mercury arc.

A Photovolt Electronic Photometer with a type A photocell was used for the intensity determination of the lamp. The type A cell is sensitive in the red and

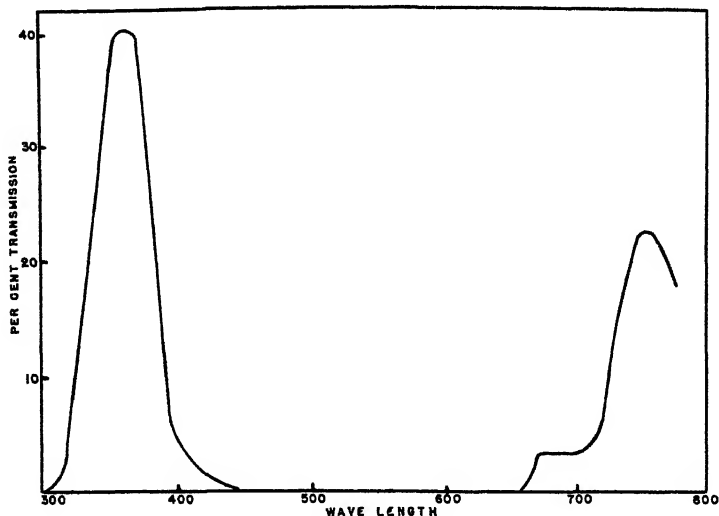


FIG. 2. Transmission of Filter SC 5022

infrared regions of the spectrum and shows no response when exposed to light below 500 mμ. A red violet H.R. type (SC 5022) filter was interposed between the cell and burner. The transmission curve of this filter was determined with a Beckman Quartz Spectrophotometer and is given in Fig. 2. By using the photometer in combination with the red violet filter, light of wave lengths of about 660 mμ and longer can be detected with no interference from the shorter wave lengths. Since the only principal line in the mercury spectrum above 660 mμ is at 690.8 mμ (McAlister, 1933), this combination of filter and light source provided a nearly monochromatic source at this wave length. This was checked by direct observation using a spectroscope.

With the photocell located in the treatment chamber with its light-sensitive portion 25 cm. from the light source, the illumination from each lamp was measured at 0.300 foot candle or  $3.23 \times 10^{-4}$  lumen/cm<sup>2</sup>.

Conversion of this value to absolute or radiation units was approximated by use of the standard observer data (Duggar, 1936). Converting lumens to watts,

$$3.23 \times 10^{-4} \text{ lumen/cm.}^2 - 63.5 \frac{\text{microwatts}}{\text{cm.}^2},$$

it was determined (see Fig. 2) that the filter transmits 3% at 690 m $\mu$ . Therefore, the total absolute intensity of this line is 2113 microwatts/cm $^2$ .

Using these data and those given by McAlister (1933) for the absolute intensities of the quartz mercury arc, the intensities of the remaining lines were calculated. The calculated results are presented in Table I. The total irradiation emitted by one of the

TABLE I  
*Spectral Distribution of Light Emitted by Hanovia Quartz Mercury Lamp*  
(SC 5010 Analytical Model)

Wave Length in m $\mu$	Spectral Region	Microwatts/cm. $^2$
690.8-390.6	Visible	378,642
365.4-289.4	Ultraviolet in sunlight	362,299
280.4-225.0	Ultraviolet not in sunlight	165,675
	Total	906,616

lamps is 907 mw./cm $^2$ . The maximum intensity that the sun commonly attains at sea level for the latitude of Washington, D. C., is 105 mw./cm. $^2$  (Duggar, 1936). Thus, in total radiation intensity, each lamp is approximately 9 times as bright as the sun. However, the difference is even greater when the ultraviolet region of the spectrum is considered alone. A maximum of 5% of the total radiation of the sun lies in the ultraviolet region of the spectrum, amounting to an intensity of 5.25 mw./cm $^2$ . The total ultraviolet light radiation intensity of each lamp is 528 mw./cm $^2$ . Thus each lamp is approximately 100 times as intense as sunlight in the region of the ultraviolet (390-225 m $\mu$ ).

Filter SC 5022 was used to filter out most of the visible and any small amount of the infrared that might be present in the radiation. The transmission curve for this filter is given in Fig. 2. To filter out the ultraviolet but permit passage of the visible light, a solution of saturated aqueous sodium nitrate was pumped through the cells in place of pure water. This solution effectively removes the ultraviolet but transmits the visible light (Duggar, 1936). Since the spectrum of the mercury discharge arc contains practically no infrared, it need not be considered here as being of any importance.

The cotton in the treatment chamber was exposed to the ultraviolet light of two lamps for a period of 4 hours. This period of exposure was selected after several preliminary trials to determine the optimum exposure which would reduce the Pressley Index of the cotton fibers to a point where an appreciable difference could be detected and yet leave enough strength in the fibers to enable considerable further reduction if such were to occur due to the action of the test organisms.

After the cotton had been exposed to the ultraviolet light for the four-hour period, it was either inoculated with *Metarrhizium glutinosum*. U.S.D.A. Beltsville 1334.2, or merely set aside as a control. Inoculation was performed by dipping the entire



frame bearing the cotton into a heavy suspension of spores of the fungus. The inoculated frame was then placed on a nutrient agar surface in a Petri dish and incubated for four days at a temperature of  $27 \pm 1^\circ\text{C}$ .

The agar substrate on which the frames were placed after inoculation was of the following composition:

Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ).....	0.5 g.
Magnesium sulfate ( $\text{MgSO}_4$ ).....	0.1 g.
Manganous chloride ( $\text{MnCl}_2$ ) .....	0.05 g.
Sodium chloride ( $\text{NaCl}$ ).....	0.1 g.
Ferrous sulfate ( $\text{FeSO}_4$ ).....	0.01 g.
Calcium chloride ( $\text{CaCl}_2$ ).....	0.02 g.
Ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ).....	2.0 g.
Agar.....	20.0 g.
Distilled water.....	to make 1 liter.

The only available carbon source was the cotton fiber itself.

At the end of the four-day growth period the frames were dipped into 95% alcohol to wash off any material which may have accumulated during incubation and to kill the fungus. After the frames had been air dried the cotton fibers were conditioned for at least 12 hours at a temperature of  $20^\circ\text{C}$ . and a relative humidity of 65%. These are the conditions of the fiber testing laboratory in which all Pressley fiber strength tests were made. The procedure followed in these tests was that recommended by Pressley (1942) and Williams and Painter (1945).

The experiments in which the irradiated cotton was inoculated with the fungus required two separate controls. The first control consisted of cotton which was irradiated but uninoculated, while the second control consisted of unirradiated inoculated cotton. Each replication of the irradiated inoculated cotton was accompanied by a sample of unirradiated inoculated fibers treated with the same inoculum to serve as a control against fluctuations in the size of the inoculum.

The cotton fibers employed in all tests reported here were samples from a large quantity of carded and combed cotton sliver of a high grade  $1\frac{1}{4}$  inch staple. This cotton was selected for its high degree of uniformity. The sliver had been extracted in a large Soxhlet-type extractor to remove all waxes, pectins, sugars and organic acids that are soluble in the commercial solvent "Solox," which contains 91% ethyl alcohol plus 5% methyl alcohol.

Since the end uses into which cotton fibers are fabricated usually require the application of various salts in the finishing processes, it appeared desirable to test the effectiveness of a couple of representative salts as catalysts for the photochemical degradation of cellulose. Consequently, cotton fibers were treated with 1% solutions of either ferrous sulfate or manganous sulfate, dried, and then exposed to the ultraviolet light.

## RESULTS

### *The Effect of Ultraviolet Light on the Pressley Indices of Cotton Fibers*

Exposure of cotton fibers to ultraviolet light was made with groups of 17 or 18 samples at a time; each group exposure was considered an

experiment. Each experiment involved a different combination of environmental conditions formed from 3 different relative humidities and 3 different oxygen concentrations. Each experiment was replicated at least 4 times and, in some cases, as many as 13 times to ensure reliable means.

Table II summarizes the data from 57 experiments run at 40°C. The data designated as "unirradiated" in Table II is that from the source material used for all experiments conducted during the course of this investigation. Its characteristics and pre-treatment have already been described in a previous section. The data presented here indicate that this starting material has an average Pressley Index of

TABLE II

*The Effect of Four-Hour Ultraviolet Light Exposure on the Pressley Indices of Cotton Fibers at 40°C. and Various Relative Humidities and Oxygen Concentrations*

Relative Humidity	Oxygen Concentration	Number of Breaks $N$	Pressley Index $\bar{x}$	Standard Error of Mean $S. E. \bar{x}$
Unirradiated Control		106	7.31	0.0258
0%	0%	95	5.80	0.0316
	21%	219	5.76	0.0470
	100%	115	5.84	0.0486
55%	0%	117	5.54	0.0304
	21%	127	5.94	0.0498
	100%	79	5.80	0.0484
80%	0%	50	5.91	0.0639
	21%	99	6.06	0.0443

7.31 with a low standard deviation of 0.264, indicating that this material was uniformly randomized. The Pressley values from which the mean was computed were not determined all at the same time from a small batch of the large sample, but were accumulated at different times during the course of the work from small batches plucked from different portions of the large randomized stock.

It is apparent from Table II that there is a large significant difference between the mean Pressley Indices of the irradiated and unirradiated control fibers. The lowest value, 5.54, for 0% oxygen concentration and 55% relative humidity shows a reduction of 24% in tensile strength from the untreated state, and the highest, 6.06, for 21% oxygen con-

centration and 80% relative humidity, shows a loss in tensile strength of 17%. The other mean values vary between these limits. Thus, there is a reduction of tensile strength of roughly 20% caused by the exposure to the ultraviolet light for 4 hours regardless of oxygen and relative humidity conditions.

*Effect of Changing Oxygen Concentration and Relative Humidity*

The data in Table II indicate that not all the mean values obtained under the various conditions of oxygen concentration and relative humidity are the same. Table III presents the calculated probabilities

TABLE III

*Probabilities that the Differences Between Mean Values Given in Table II are Due to Errors in Chance Sampling*

Relative Humidity	0%				55%			80%
	Oxygen conc.	0%	21%	100%	0%	21%	100%	0%
0%	21%	0.48						
	100%	0.48	0.24					
55%	0%	$10^{-8}$	$10^{-4}$	$10^{-4}$				
	21%	0.38	$10^{-2}$	0.15	$10^{-6}$			
	100%	1.00	0.57	0.56	$10^{-6}$	0.04		
80%	0%	0.13	0.62	0.37	$10^{-6}$	0.72	0.17	
	21%	$10^{-6}$	$10^{-6}$	0.65	$10^{-8}$	0.07	$10^{-8}$	$10^{-3}$

that the various differences between the mean values are due to errors in sampling. It is evident from these data that there are significant differences between some of these values, but not all. Since there is no consistency in the way in which the means vary, however, no conclusions can be drawn here concerning the effect of different oxygen concentrations and relative humidities other than that there does not appear to be any marked effect which is measurable under the conditions of these experiments.

*Growth of Metarrhizium glutinosum on Irradiated  
and Unirradiated Cotton*

Each irradiated group of fibers was inoculated along with an equal number of unirradiated groups and incubated for 4 days under the same conditions. After incubation the fibers were broken and the Pressley Indices tabulated. The differences between the indices of the irradiated and unirradiated inoculated samples were determined and the mean differences for each experiment calculated. The means of 72 experiments are tabulated in Table IV together with the respective standard errors.

TABLE IV  
*The Difference Between the Tensile Strength of Irradiated and Unirradiated  
Cotton After Four Days Growth of Metarrhizium*

Relative humidity	Oxygen concentration	Number of breaks	Mean difference	Standard error	Difference between irradiated and unirradiated uninoculated	Total difference in loss of strength expressed as Pressley Indices
0%	0%	147	+0.38	.0403	1.51	1.89
	21%	165	+0.44	.0509	1.55	1.99
	100%	155	+0.47	.0391	1.47	1.94
55%	0%	57	+0.51	.0673	1.77	2.28
	21%	113	+0.40	.0749	1.37	1.77
	100%	42	+0.45	.0555	1.51	1.96
80%	0%	60	+0.47	.0249	1.40	1.87
	21%	57	+0.45	.0264	1.25	1.70

It is to be noted that all the mean differences are designated as being positive in Table IV. In calculating the differences in Pressley Indices between the unirradiated and irradiated inoculated fibers, the differences were taken to be positive when the unirradiated fiber index was lower than the irradiated fiber index. Therefore, these data show that, in all cases, the mean tensile strength of the irradiated fibers was higher than in the case of the unirradiated fibers after 4 days growth of the fungus. Since the unirradiated fibers had a higher Pressley Index initially than the irradiated fibers, the strength loss curves for both cases made by plotting Pressley Index against fungus growth period must cross at some point prior to 4 days. There are not enough data to determine these curves exactly. However, Fig. 3, based on the average data, presents the idea graphically. Here two lines designating loss in

weight by fungus activity on the unirradiated and irradiated fibers are drawn, each being determined by 2 points, the value of the Pressley Index at 0 time and the approximate value 4 days later. It can be seen from Fig. 3 that the average strength loss was almost twice as great for the unirradiated fibers as for the irradiated fibers. Thus, during the first 4 days of fungus growth the degradation of the unirradiated fibers proceeds at an average rate twice that of the degradation of the irradiated fibers.

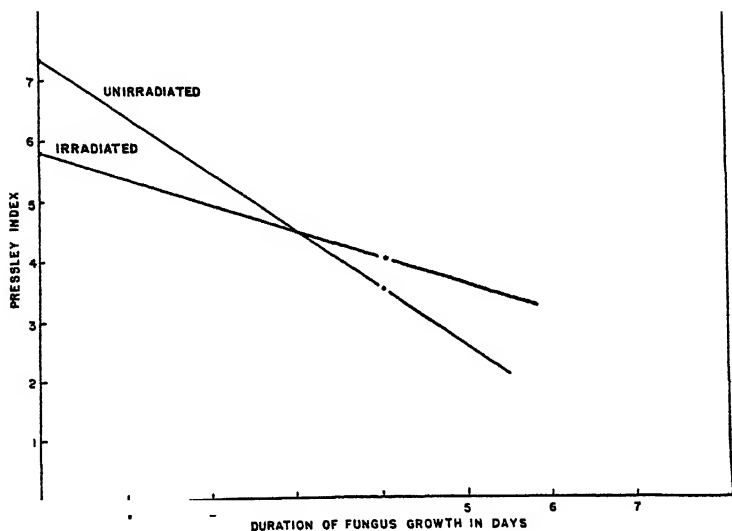


FIG. 3. Growth of *Metarrhizium glutinosum* on Cotton Fibers Irradiated and Unirradiated with Ultraviolet Light

In column six of Table IV there are listed the differences between the values of the uninoculated irradiated fibers and the uninoculated unirradiated fibers given in Table II. These are the differences in Pressley Indices for the variously treated fibers at the beginning of fungal growth. The sums of these values and the corresponding mean differences at the end of 4 days give the total difference in loss of strength at the end of 4 days. These data are given in column seven of Table IV. It can be seen that the total difference in strength loss was approximately 2 Pressley units for all conditions. Apparently, the fungus attacked the fibers exposed to light under the various conditions with

approximately the same effect. This conclusion is consonant with the conclusion arrived at in the previous section, *i.e.*, that the effect of the ultraviolet light on the tensile strength of cotton fibers is not demonstrably influenced by the oxygen concentration and relative humidity during a four-hour period of exposure.

### *Effect of Temperature*

All experiments which have been described up to this point were performed at a temperature of 40°C. with 2 ultraviolet lamps. Only the oxygen concentration and relative humidity were varied. To test the influence of temperature on the effect of ultraviolet light on cotton fibers, a series of experiments were performed at a temperature of 15°C., maintained in the chamber by the methods described above. The relative humidity was maintained at approximately 50% and the oxygen concentration was kept at 21%. Due to the difficulties involved in maintaining a control over relative humidity with the water bath at 0° and the chamber at 15°C., and since it has been demonstrated in the previous sections that there is no apparent effect of relative humidity, we have chosen to employ the data obtained from previous experiments run at 80% relative humidity as the control for this experiment.

TABLE V  
*Effect of Low Temperatures on Loss of Strength Due to Exposure to  
Ultraviolet Light for Four Hours*

Temperature	Number of breaks	Mean P.I.	S. E.
15°C.	76	5.95	0.0213
40°C.	160	5.74	0.0683

Table V summarizes these data, which indicate that there is a difference between the tensile strengths after exposure to the ultraviolet light for 4 hours. The probability that this difference is due to chance alone is about one in one thousand. It can be concluded, then, that lowering the temperature appears to slow the photochemical reaction(s).

### *The Effect of Photochemical Catalysts*

Table VI summarizes the data from experiments in which cotton fibers were treated with iron and manganese salts before exposure to the ultraviolet light. These experiments were performed at 40°C. with a relative humidity of about 80% and an oxygen concentration of 21%. The data indicate that the manganese and iron salts do catalyze the

TABLE VI  
*Effect of Photochemical Catalysts on Loss of Tensile Strength Due to Exposure to Ultraviolet Light*

Catalyst	Number of breaks	Mean P.I.	S. E.
FeSO <sub>4</sub>	82	5.43	0.0555
MnSO <sub>4</sub>	80	5.25	0.0531
Control	160	5.74	0.0683

photochemical reaction of cellulose in the presence of ultraviolet, since the Pressley Indices for the treated fibers are both significantly lower than the index representing the control.

*Relative Effectiveness of Ultraviolet and Visible Wave Lengths in Decreasing Tensile Strength of Fiber*

Table VII presents the results of experiments in which cotton fibers were exposed to those portions of the mercury discharge spectrum passed by filter SC 5022 (mainly 360–370 m $\mu$ ; see Fig. 2) and by a saturated solution of sodium nitrate. These experiments were performed at a temperature of 40°C., an oxygen concentration of 21%

TABLE VII  
*Effect of Filtering out Regions of the Mercury Discharge Arc Spectrum*

Spectral region	Number of breaks	Mean P.I.	S. E.
360–370 m $\mu$	65	6.52	0.0875
Visible	22	7.05	0.0905

and a relative humidity of 55%. The data indicate that the principal, if not the complete, effect of light on cotton is caused by the ultraviolet light and not the visible light band of the spectrum. It is to be noted that, since the filter passed only about 40% of the total intensity of the 365.4 m $\mu$  line of the mercury arc, the intensity of irradiation from one lamp with the filter was only approximately 63.4 mw./cm<sup>2</sup>. This should be contrasted with the intensity of the visible light from the lamps which, even with the sodium nitrate solution as a filter, approached 379 mw./cm.<sup>2</sup> intensity.

## DISCUSSION

The evidence derived from the experiments presented in this report is in agreement with the data cited by previous workers and shows further that exposure to the 360–370  $m\mu$  range alone reduces the tensile strength of cotton fibers as much as exposure to the entire spectrum of the mercury vapor arc. Therefore, in this discussion it is assumed that the visible light from the lamps used had little or no effect on the observed tensile strength losses which are due rather to the ultraviolet light emitted by the lamps.

There exists considerable controversy in the literature over the role played by oxygen in the deterioration of cellulose by the ultraviolet light. Barr and Hadfield (1928) found the presence of free oxygen to be necessary during the exposure period, while, on the other hand, Kaufman (1926) and Heuser (1943) present evidence to show that the deterioration of cellulose can take place in the absence of oxygen. The main point made by both Kaufman and Heuser is that the preliminary reaction under the influence of ultraviolet light is a true photochemical reaction and no free oxygen is involved. This initial reaction is followed by a second reaction in which free oxygen plays a part and oxycellulose is formed as a result. There has been a claim made that the initial (photochemical) reaction does not reduce the tensile strength but rather, under certain conditions, an increase in strength can be noted, and a patent has been issued for increasing the tensile strength of cellulose fibers by controlled ultraviolet exposure in which no oxycellulose is formed (Pacini, 1934).

The work of Stillings and Van Nostrand (1944) demonstrates that there is almost certainly a photochemical reaction occurring during exposure which does give a reduction in the degree of polymerization as determined by viscosity measurements, a reduction of  $\alpha$ -cellulose content, an increase in copper number and a liberation of CO and CO<sub>2</sub>, and that this reaction occurs in the complete absence of oxygen. This is followed by an oxidation of the ultraviolet-treated cellulose in the presence of free oxygen with the formation of oxycellulose. This latter oxidation can be arrested at any time by placing the sample back in nitrogen, only to resume when the sample is again returned to oxygen.

If we accept the interpretations of Stillings and Van Nostrand, then the confusion of earlier workers in the field is explainable. If this interpretation is followed, it leads to the conclusion that the photochemical



reaction is the limiting one and free oxygen can only act on the exposed cotton until all of the photochemically altered portions have been oxidized, whereupon the oxidation must cease, since atmospheric oxygen apparently does not affect native unaltered cellulose.

Heuser and Chamberlin (1946) found that irradiation of cellulose triacetate in nitrogen did not increase the reducing power as it did with cellulose, and that the acetyl content remained unchanged. This material also manifested a pronounced post-irradiation effect which indicates that the hydroxyl groups in cellulose are not involved in the photochemical reaction which predisposes the cellulose to chain length reduction in the presence of oxygen.

The data presented in this report demonstrate that there is no apparent effect of the oxygen concentration in the environment during the period of exposure. When it is realized that the tensile strength of the fibers was not determined immediately after exposure, but rather several days to weeks afterwards, it becomes apparent that Stillings and Van Nostrand's explanation might apply here. During the period before breaking, the fibers were all conditioned for several days in the atmosphere—in other words, in the presence of 21% oxygen. In this way, since all samples were exposed to the ultraviolet light for the same period, they were all subsequently oxidized by free oxygen to approximately the same extent and, as a result, lost about the same amount of tensile strength. This interpretation might also explain the rather high degree of variability in the Pressley Indices, since the fibers were conditioned for varying periods before testing on the Pressley apparatus.

Further investigation of the photochemical reaction(s) and the subsequent oxidation of ultraviolet-irradiated cellulose will be undertaken to determine the nature of the changes which cause a reduction of the "degree of polymerization" of the cellulose and a loss in tensile strength of cotton fibers. This work might indicate a surface treatment of cotton fibers which will protect them from degrading effects of sunlight by filtering out the effective wave lengths or cover the reactive groups of the cellulose molecule.

The effect of the moisture content of the fibers during exposure to the ultraviolet light is usually assumed to be such as to aid the deterioration process. This is not supported by a great deal of evidence, and certainly the evidence does not seem to be conclusive. Barr and Hadfield (1928) have stated that they have been able to demonstrate

a difference in the rate of deterioration due to ultraviolet light in a dry and a moist atmosphere, while Heuser (1943) states that the presence of moisture is not essential for the photochemical reaction according to the results obtained in his laboratory. The data reported here do not indicate that there is any significant difference between tensile strengths of cotton fibers irradiated at high and low relative humidities. However, as in the case of oxygen concentration, the effect may not be detectable, except during the first stages of the irradiation, since the photochemical reaction is the limiting factor. All subsequent reactions which proceed, although affected by atmospheric conditions, ultimately lead to the same end result as far as tensile strength is concerned.

The effect of temperature demonstrated by the results presented in the preceding sections is in agreement with the work of others (Pytser, 1923, and Stillings and Van Nostrand, 1944), who have shown that an increase in the exposure temperature brings about an increase in the reaction rate. This is certainly to be expected, since the rate of photochemical reactions like that of any other chemical reaction is dependent in part on temperature.

The data presented in Table IV indicate that the difference between the tensile strengths of irradiated and unirradiated cotton fibers after 4 days growth of *Metarrhizium* is in all cases positive, i.e., the irradiated cotton fibers lost less tensile strength by fungal decay than did the unirradiated. Therefore, it may be stated that there is no predisposition to biological degradation induced in cotton fibers by ultraviolet light irradiation, but rather that there is a slight resistance to fungal action imparted by the irradiation. This effect may be accounted for on the basis of an alteration of the cellulose from its native state due to the photochemical reaction and the subsequent oxidation. The modified cellulose thus formed presents a substrate different from the native cellulose which, in turn, will affect the nature and rate of fungal growth. This hypothesis is in harmony with the demonstration (unpublished results) that oxidized cellulose derivatives such as 6-carboxy cellulose and cellulose-2,3-dialdehyde are more resistant to *Metarrhizium glutinosum* than cellulose itself.

#### ACKNOWLEDGMENTS

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## SUMMARY

1. The Pressley Index of tensile strength of cotton fibers has been demonstrated to be a useful and convenient measure of the effect of ultraviolet irradiation and fungal action on cotton fibers.

2. As measured by the Pressley Index, the loss of tensile strength of cotton fibers due to action of ultraviolet light is independent of oxygen concentration and humidity, but is positively correlated with ultraviolet light intensity and temperature. There is a reduction in fiber tensile strength of approximately 20% after 4 hours of exposure to ultraviolet radiation of an intensity of 1060 mw./cm<sup>2</sup>.

3. Iron and manganese salts catalyze the degrading effect of ultraviolet light on cotton cellulose.

4. The ultraviolet portion of the spectrum, as compared to the visible band, is most effective in modifying the cotton fiber as measured by changes in tensile strength.

5. Exposure of cotton fibers to ultraviolet light increases their resistance to subsequent attack by *Metarrhizium glutinosum*. During the first 4 days of fungal growth the average rate of degradation of the unirradiated fibers was twice that of the irradiated fibers.

6. There was no difference in the rate of fungal attack on fibers which were irradiated for the same length of time and with the same light intensity but with different oxygen concentrations and humidities.

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# Changes in the Essential Amino Acid Content of the Proteins of Dry Skim Milk on Prolonged Storage

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## INTRODUCTION

Henry and Kon (1) have reported that the biological value of the proteins of a sample of dry skim milk decreased on prolonged storage. Their report covers the storage period from 19 to 54 months and shows a decrease from a biological value of 88.5 to 71.1 during this period. The high biological values reported after 19 months suggests that any early storage decrease must have been small or non-existent. Mitchell (2) has reported decreases, on storage, in the biological value of the proteins of soybeans and corn, and Jones, Divine and Gersdorff (3) have shown that the feeding value of corn protein decreases on storage.

Since it seemed that the decrease in biological values may indicate a loss of essential amino acids during storage, we have investigated the amino acid content of the proteins in stored dry skim milk in comparison with that in recently processed dry skim milk. Our results, however, do not necessarily give a complete explanation of the loss of biological value since factors other than amino acid content contribute to the biological value. The latter fact has been discussed by Mitchell (2) and further elucidated by Melnick, Oser and Weiss (4). Mitchell and Block (5) have recently reviewed the relationships between amino acid contents of proteins and their nutritive value for the rat.

## SAMPLES

While the study of storage losses of nutritive values is best carried out by analyses of a sample of the fresh product and similar analyses on the same sample after storage, such a procedure requires the lapse of considerable time before completion of the investigation when prolonged storage periods are involved. An alternative procedure involving the comparison of fresh and stored samples has been used in this investigation.

This procedure offers some advantages, particularly when microbiological assay methods are used, since comparisons are made to the same standards and differences in environmental conditions can be minimized. For this investigation, samples from the same plant were paired and the paired samples were placed side by side in the autoclave and incubator and were manipulated consecutively for dilutions, titrations, etc.

The stored samples used were approximately 51 months old when analyzed. The stored samples were all stored in the laboratory at room temperature. They were all white high quality samples before storage. The analyses on the fresh samples were made within a month and a half to two months after processing. All samples were spray-dried.

The first two pairs of samples were from a plant in Wisconsin. The old samples of these pairs were stored in tin cans with sleeve-type tin covers loose enough to permit some interchange with the surrounding air. These stored samples formed into large, rather hard lumps, had markedly darkened in color and had an "old," slightly unpleasant odor and taste. The fact that these samples were not packed in tight cans and were thus exposed to atmospheric oxygen and moisture may have contributed to these changes. The reasons for the changes in the stored samples are not well understood, but are very important since they affect not only the palatability but also the nutritive value, as will be shown in the results of this investigation.

The last three pairs of samples were from a plant in Utah. The old samples in this series were packed in tin cans with double-friction type lids that rather effectively excluded atmospheric oxygen and moisture other than that enclosed in the container. These samples retained the normal white color of high quality dry skim milk powder and were quite similar to the fresh powders in appearance, odor and taste.

The percentages of protein in the samples, as calculated by multiplying the micro Kjeldahl nitrogen by 6.38, are as follows: Pair 1, old 34.54, fresh 33.97; Pair 2, old 34.99, fresh 34.38; Pair 3, old 37.30, fresh 36.91; Pair 4, old 36.20, fresh 36.33; and Pair 5, old 36.78, fresh 37.52.

## METHODS OF ASSAY

Microbiological assays for all the essential amino acids except phenylalanine were conducted by the method of Stokes, Gunness, Dwyer and Caswell (6). For the phenylalanine and tyrosine assays we have used *Leuconostoc mesenteroides* P-60 with the media of Dunn, Shankman, Camien, Frankl and Rockland (7). The use of this organism for phenylalanine and tyrosine assays of milk protein has been reported by Hodson and Krueger (8). For the phenylalanine assays the acid hydrolyzate prepared by the method of Stokes, Gunness, Dwyer and Caswell (6) was used, and for the tyrosine assays, the sodium hydroxide hydrolyzate proposed by the same investigators was employed. The successful use of the sodium hydroxide hydrolyzate for tyrosine assays with *L. delbrückii* has been reported by Gunness, Dwyer and Stokes (9). The reason tyrosine was included in the investigation was because it may exert a sparing action on phenylalanine. Arginine was included because it is essential for maximum growth in some species.

## RESULTS

The results of the microbiological assays for the various amino acids in each sample are compiled in Table I. Inspection of these data indicate

TABLE I  
*Amino Acid Content of the Proteins \* of Dry Skim Milk Which Had Been  
Stored for Four Years and Freshly Processed Dry Skim Milk*

	Sample pair 1		Sample pair 2		Sample pair 3		Sample pair 4		Sample pair 5	
	<i>Per cent amino acid of protein</i>		<i>Per cent amino acid of protein</i>		<i>Per cent amino acid of protein</i>		<i>Per cent amino acid of protein</i>		<i>Per cent amino acid of protein</i>	
	Old	Fresh	Old	Fresh	Old	Fresh	Old	Fresh	Old	Fresh
Arginine	2.68	3.18	2.80	2.93	2.78	3.09	3.48	3.29	3.12	3.14
Histidine	1.76	2.25	1.83	2.22	2.35	2.39	2.68	2.45	2.31	2.29
Isoleucine	5.53	5.80	5.74	5.76	5.88	5.82	6.46	6.40	6.66	6.40
Leucine	9.33	9.64	9.35	9.70	9.64	9.52	10.05	10.17	9.82	9.90
Lysine	5.73	7.42	6.15	7.77	7.66	7.86	7.91	8.44	7.75	7.68
Methionine	2.12	2.41	2.11	2.41	2.39	2.44	2.46	2.67	2.50	2.45
Phenylalanine	5.19	5.50	4.92	5.00	5.27	5.43	5.64	5.62	5.63	5.20
Threonine	3.71	4.01	4.06	4.05	3.90	4.03	4.28	4.05	4.21	4.11
Tryptophan	1.15	1.30	1.27	1.35	1.37	1.40	1.48	1.49	1.41	1.40
Tyrosine	4.80	4.85	4.67	4.88	4.98	4.97	5.32	5.32	5.30	5.12
Valine	5.62	5.88	5.94	5.76	5.90	5.74	6.43	6.33	6.31	6.32

\* Micro Kjeldahl nitrogen multiplied by 6.38.

a loss of arginine, histidine, lysine and methionine. The data further suggest that these losses are almost entirely confined to the old samples which had become dark and unpalatable. In order to illustrate the latter suggestion, the differences between the two paired samples in *per cent* have been calculated and the results averaged according to whether the stored samples became discolored or remained white. The results of the calculations (Table II) show that stored samples which have become discolored have suffered marked losses of arginine, histidine, lysine and methionine, a somewhat smaller loss of tryptophan and minor and insignificant losses of isoleucine, leucine, phenylalanine, threonine, tyrosine and valine. In contrast, the average of the comparisons between pairs in which the old sample retained its white color show only minor and probably insignificant differences.

TABLE II  
*Differences in Amino Acid Content between Old and Freshly Processed  
 Dry-Skim Milk Proteins*

	Average difference of pairs in <i>per cent</i> <sup>1</sup> where old sample was discolored (Pairs 1 and 2)	Average difference of pairs in <i>per cent</i> <sup>1</sup> where old sample remained white (Pairs 3, 4 and 5)	Average <i>per cent</i> <sup>1</sup> difference of all five pairs
Arginine	-10.0	-1.2	- 4.8
Histidine	-19.6	+2.9	- 6.1
Isoleucine	- 2.5	+2.0	+ 0.2
Leucine	- 3.4	-0.2	- 1.5
Lysine	-21.8	-2.6	-10.5
Methionine	-12.2	-2.7	- 6.5
Phenylalanine	- 3.5	+1.8	- 0.7
Threonine	- 3.6	+1.9	- 0.5
Tryptophan	- 8.7	-0.7	- 4.5
Tyrosine	- 2.7	+1.2	- 0.2
Valine	- 0.6	+1.4	+ 0.6

\* The amino acid content of the fresh sample is considered 100. A minus sign indicates the old sample contained less of the amino acid.

The inclusion of cystine in the list of amino acids for which assays were made would have been of value, for while cystine is not an essential amino acid it exerts a sparing action on methionine. A satisfactory microbiological method for cystine that can be directly applied to milk has not yet been found. One problem is the preparation of a suitable hydrolyzate for the assay, since, in the presence of carbohydrate, cystine is lost on both acid and alkaline hydrolysis, as is mentioned by Block and Bolling (10). The use of special cystine-conserving methods of hydrolysis such as those used by Hess, Sullivan and Palmes (11) has not been investigated but use of these procedures might considerably complicate the use of a microbiological method.

The possibility of loss of other amino acids in the preparation of hydrolyzates has been mentioned in an earlier publication of Hodson and Krueger (8) and references to more detailed information were given.

#### ACKNOWLEDGMENTS

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### SUMMARY

1. The content of essential amino acids, arginine and tyrosine of the proteins of five dry skim milk samples which had been stored at room temperature for 51 months was compared with that of paired samples of recent manufacture.

2. Two of the stored samples which had darkened in color and become less palatable lost arginine, histidine, lysine, methionine and a smaller amount of tryptophan.

3. In contrast, the three stored samples which retained their fresh appearance and taste suffered no significant losses of essential amino acids.

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# On the Mode of Action of Penicillin \*

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## INTRODUCTION

The inhibitory action of impure preparations of penicillin on the dismutation of pyruvic acid by *Staphylococcus aureus* was previously reported (1). Subsequent purification of these penicillin preparations showed that the inhibitory properties lay in the impurities. This phenomenon was not further investigated since the primary interest was in the various penicillins. In a preliminary report an inhibitory effect by crystalline penicillin G on the metabolism of nucleic acid and/or nucleotides was reported (2). The purpose of this communication is to extend this work.

Reports by other investigators (3) that penicillin had no deleterious effect on the metabolism of glucose were confirmed. Extensive experiments using both enzyme preparations and intact cells of *Staph. aureus* showed that the antibiotic had no effect on enzymes concerned with the intermediate products of carbohydrate metabolism.

An attempt was then made to study the effect of penicillin on the metabolic activity of a mass inoculum on the chemically defined medium employed by Fildes *et al.* (4) which satisfies the nutritional requirements of *Staph. aureus*. A mass inoculum was used to obtain differences in metabolic reactions sufficiently large to measure. Such differences are difficult to obtain in ordinary growth experiments. Criteria used to measure metabolic changes were oxygen uptake, carbon dioxide liberation (anaerobically and aerobically) and the production or utilization of acid in bicarbonate buffer in the presence or absence of oxygen. There was apparently no effect of penicillin on the

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metabolism of the medium employed as an entity, or on any of the individual constituents, or on various combinations of the constituents, when measured during a period of 1-5 hours. Invariably in the absence of penicillin a gradual acceleration of oxygen uptake would begin after 5 hours reaching a relatively high maximum in some cases as early as 6 hours, and in others as late as 9 hours depending upon the condition of the mass inoculum. In the presence of penicillin this acceleration did not occur. Control experiments showed that the same phenomenon occurred with cells and phosphate buffer, and that apparently the substance or substances being oxidized were constituents of the cells.

That the antibiotic was not exhibiting a general toxic effect owing to its prolonged contact with the cells was demonstrated by the addition of glucose just prior to the rapid acceleration period. The glucose was metabolized immediately as judged by oxygen uptake, and the increased metabolism was not inhibited by penicillin. In addition, when the exposure of the cells to penicillin was continued until the penicillin was destroyed by lability, the cells showed a typical acceleration of oxygen uptake. The penicillin did not irreversibly denature any of the proteins of the enzyme systems involved in the activity.

To elucidate the nature of this endogenous metabolism, the products were determined and were found to be carbon dioxide and acetic acid. The ratio of oxygen utilized to carbon dioxide liberated to acetic acid formed was 3:3:1. This relationship may be expressed by the following equation:



The equation represents the oxidation of a pentose with a respiratory quotient of one and the accumulation of acetic acid.

Dickens (5) found that ribose or ribose-5-phosphate is not metabolized by intact cells of yeast; however, if Lebedev juice from yeast was employed, ribose-5-phosphate was rapidly metabolized. Apparently the phosphorylated sugar cannot permeate the membrane of the intact cell and, under conditions of the experiment, ribose cannot be phosphorylated. Neither ribose nor ribose-5-phosphate is metabolized by intact cells of *Staph. aureus* and as yet we have been unable to secure an active enzyme preparation. During the course of rapid endogenous activity, referred to above, pentose disappears; the presence of penicillin inhibits this disappearance. We believe the source of

pentose (ribose) to be ribonucleic acid (R.N.A.). In some as yet undetermined manner penicillin interferes with the dissimilation of ribonucleic acid and consequently with its assimilation during growth.

### METHODS

The organism usually employed in these studies was *Staph. aureus*. Similar results were obtained with *Micrococcus lysodeikticus* and *Lactobacillus casei*.

The staphylococci were inoculated into a medium consisting of 1% glucose, 0.5% peptone (Difco), 0.4% yeast extract, 1.5%  $K_2HPO_4$  and 10% tap water. The last two constituents were autoclaved separately. The inoculated medium was incubated at 37°C. and the cells were harvested after 24 hours. Owing to the large amount of acetic acid produced during growth, the medium must be heavily buffered, otherwise cells will be obtained which are relatively inactive when placed on a variety of substrates. Freshly harvested cells were generally employed in these experiments; however, it was found that such cells could be lyophilized without any change in their behavior. Most of the experiments were conducted in conventional Warburg flasks. In experiments where larger quantities of material were required for analyses, 125 ml. flasks were attached to the manometers.

To conserve the crystalline sodium salt of penicillin type G, preliminary experiments were conducted with commercial preparations whose purity ranged from 800–1000  $\gamma$ /mg. Whenever it was apparent that an impurity in the commercial preparation was interfering, the crystalline product was used.

Respiratory quotients were determined by the customary differential method. Carbon dioxide was also determined gravimetrically after its release from the filter paper by acid. Acetic acid was determined by the partition method of Osburn *et al.* (6).

In some experiments cells were incubated in phosphate buffer until the acceleration period was about to begin. These cells were centrifuged and the paste employed as directed in the tables.

Schneider (7) has reported that nucleic acids can be quantitatively extracted from tissue with 5% trichloroacetic acid (T.C.A.) at 90°C. for 15 minutes. We have found that 2 similar extractions of bacterial cells are required to quantitatively remove the nucleic acids from the nucleoproteins. In an attempt to determine whether penicillin was interfering with the nucleic acid, nucleotide or nucleoside stage, fractionations of these components were made with uranyl acetate at pH values recommended by Macfayden (8) and Kerr (9). No significant conclusions could be drawn from these data.

Details of the hot T.C.A. extraction are as follows: To every ml. of bacterial suspension (containing 10 mg. lyophilized cells) to be analyzed, 5 ml. of 6% T.C.A. were added. For each time period 2 such samples were prepared, A and B. To determine the total R.N.A., sample A was heated at 90°C. for 15 minutes and centrifuged. The supernatant liquid was separated and the cells suspended in 4 ml. of 5% T.C.A. and the heat treatment repeated. The supernatant liquids which contain the total nucleic acids of the cells were combined and made up to 10 ml. for ribose determinations.

Sample B was centrifuged immediately and the supernatant saved. The cells were extracted with hot 5% T.C.A. as described above to remove the nucleic acids

associated with the cells. The cold and hot T.C.A. supernatant liquids were made to 10 ml. for ribose determinations.

The nucleic acid content of these various fractions was followed by the orcinol determination of ribose (Mejbaum, 10). The pyrimidine nucleotides are not quantitatively determined by this method, however no attempt has been made to determine the absolute quantity of ribonucleic acid but rather relative amounts in the presence and absence of penicillin. Desoxyribonucleic acid was determined by the diphenylamine reaction (Dische, 11). Since this determination is not as sensitive as the orcinol reaction for ribonucleic acid, the final dilutions after T.C.A. extractions were 1:3. Aliquot samples were used for the determination.

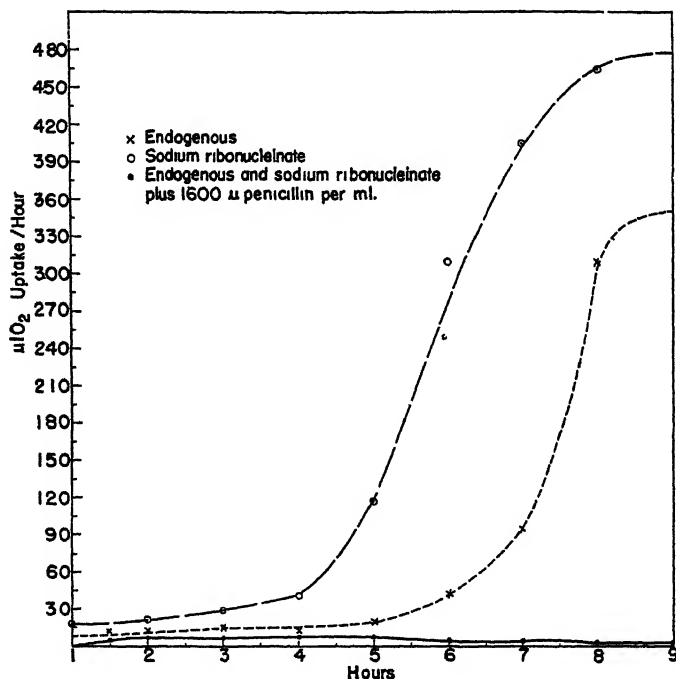


FIG. 1. Total Volume 2 ml. Containing 40 mg. Lyophilized Cells. 0.25 mM  $\text{PO}_4$  Buffer pH 7.0 1.5% Sodium Ribonucleinate.

### EXPERIMENTAL

Fig. 1 reveals the oxygen uptake exhibited by the endogenous metabolism over a period of 9 hours. In the absence of penicillin the endogenous oxygen uptake was maintained at a constant small level for 5 hours when the rapid acceleration occurred. In the presence of peni-

cillin the acceleration did not occur. Fig. 1 also shows the oxygen uptake with sodium ribonucleinate as the substrate. The period of lag before acceleration was shortened and the rate of oxygen uptake was greater than the endogenous activity. Penicillin completely inhibited the metabolism of the added sodium ribonucleinate.

The similarity between the curves representing sodium ribonucleinate and the endogenous activity suggests that the substrate oxidized in the cells was ribonucleic acid.

Apparently during the lag period there was a gradual accumulation of an oxidizable substrate until the enzyme systems were saturated. When the organisms were incubated in phosphate buffer until the acceleration attained a maximum or near maximum value, the addition of penicillin caused very little immediate inhibition of the oxygen uptake. Later a definite inhibition occurred which could be interpreted as a depletion of the substrate which is being oxidized and an inhibition of the further release of this substrate by the antibiotic. If, however, penicillin was added just prior to the acceleration period the degree of inhibition was as great as when the penicillin was added at zero time.

The fact that the ratio of oxygen uptake:carbon dioxide liberated:acetic acid produced was 3:3:1 during the endogenous metabolism and that the same ratio existed when sodium ribonucleinate was used as the substrate, further substantiates the hypothesis that the endogenous activity is related to nucleic acid metabolism (*cf.* Table I).

To show that the effect of penicillin on the endogenous oxygen uptake was not general toxicity but rather inhibition of a specific metabolic process, glucose was added as a substrate to experiments with and without penicillin after the lag period had continued for 4 hours (Fig. 2). In both experiments there was an immediate accelerated oxygen

TABLE I  
*Comparison of Oxygen, Carbon Dioxide and Acetic Acid During Dissimilation*

	Millimoles				
	O <sub>2</sub>	CO <sub>2</sub>	CH <sub>3</sub> COOH	R.Q.	O <sub>2</sub> :CO <sub>2</sub> :CH <sub>3</sub> COOH
Endogenous	0.661	0.659	0.224	0.99	3:3:1
Sodium ribonucleinate	1.520	1.531	0.521	1.01	3:3:1

125 ml. Warburg flask containing 600 mg. lyophilized cells, 4.0 mM PO<sub>4</sub> buffer, pH 7.0. Sodium ribonucleinate 1.5%. Total volume 30 ml.; temp., 30°C.; time, 24 hrs.

TABLE II

*Ribonucleic Acid Content of Cells after Hot and Cold T.C.A. Extraction*

		Zero time		24 Hours	
		No pen.	Pen.	No pen.	Pen.
Sample A	Supernatant liquid	20	53	73	198
	Cells	210	195	28	45
	Total	230	248	101	243
Sample B	Total hot T.C.A. Extraction	240	250	100	240

Data expressed in  $\gamma$  ribose/10 mg. cells, 125 ml. Warburg flasks containing 300 mg. lyophilized cells. 0.5 mM  $\text{PO}_4$  buffer, pH 7.0. 2000 u. penicillin/ml.

Total volume, 30 ml. Temperature 30°C.

Sample B. Supernatant liquid represents cold T.C.A. extraction. Cells extracted with hot T.C.A.

Sample A. Cells extracted with hot T.C.A. See text.

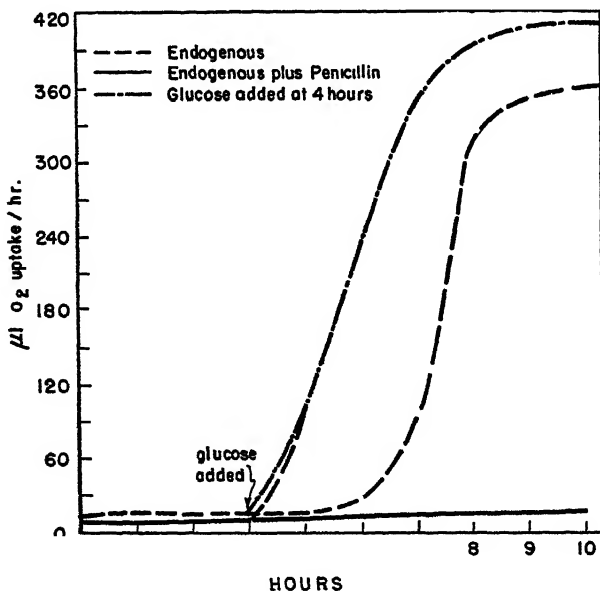


FIG. 2. Total Volume 2 ml. Containing 40 mg. Lyophilized Cells. 0.25 mM  $\text{PO}_4$  Buffer pH 7.0. 0.05 mM Glucose Added. 1000 u. Penicillin/ml.

uptake, the penicillin being without influence on the activity. Without the addition of glucose the acceleration occurred in the typical manner in the absence of penicillin and showed the usual inhibition in its presence.

Most of the nucleic acids are associated with the cellular material at zero time. Compare the amounts of these acids in the cold T.C.A. supernatant liquid and the amount obtained from the cells after hot T.C.A. extraction in sample A. After 24-hour metabolism the nucleic acids are dissociated from their proteins and, in the presence of penicillin, none has been metabolized. In the absence of penicillin their content has fallen materially.

In sample B, Table III, the determination of the ribonucleic acid was made by direct hot T.C.A. extraction without carrying out the initial cold T.C.A. extraction. These values agree closely with the totals obtained in sample B.

TABLE III

*Disappearance of Added Sodium Ribonucleinate during Dissimilation*

Zero time		24 Hours	
No pen.	Pen.	No pen.	Pen.
1245	1328	415	1260

Data expressed in  $\gamma$  ribose/ml. Conditions as in Table II with 1.5% sodium ribonucleinate added.

Table III shows the inhibitory effect of penicillin on the dissimilation of sodium ribonucleinate added as substrate. The values represent both the added ribonucleinate and the ribonucleic acid normally present in the cells. In the absence of penicillin there was a decided decrease of the ribonucleinate at the end of the metabolic period, whereas in its presence the decrease was insignificant.

The decrease in nucleic acid appears to be only in the ribose type. The desoxyribose type does not decrease during metabolism (Table IV). Since there was no decrease in the latter, results are expressed only in colorimeter readings of the diphenylamine reaction.

The mononucleotides representing the components of yeast nucleic acid were likewise tested for the penicillin effect (Table V). Guanylic acid apparently was not dissimilated by the cells since the oxygen-uptake in its presence was approximately the same as that of the endogenous or of the yeast adenylic acid. The mixture of the pyrimidine nucleotides, cytidylic and uridylic acids, isolated by the procedure of



TABLE IV  
*Desoxyribonucleic Acid Content of Cells*

	Colorimeter readings		
	0 Hrs.	15 Hrs	24 Hrs.
No penicillin	116	111	108
Penicillin	112	115	112

Conditions as in Table II. Final dilution of 5% T.C.A. extract was 1:3.

TABLE V  
*Effect of Penicillin on Components of Ribonucleic Acid*

	No pen.	$\mu$ l. O <sub>2</sub>	Pen.
Endogenous	699		176
Sodium ribonucleinate	1871		510
Guanylic acid	792		287
Pyrimidine nucleotides	1198		364
Yeast adenylic acid	711		234

Total volume 2 ml. containing 40 mg. lyophilized cells previously incubated in phosphate buffer. 0.25 mM PO<sub>4</sub> buffer, pH 7.0. 1600 u. penicillin/ml. Sodium ribonucleinate 1.5%. Nucleotides in equivalent amounts. Time, 10 hours. Temperature, 30°C.

Levene and Bass (12), showed an excess oxygen-uptake which was significantly larger than the endogenous. It was also inhibited by penicillin. However, the values obtained for these pyrimidine nucleotides do not approach the sodium ribonucleinate from which they were isolated.

Table VI shows quantitative effects of penicillin on 40 mg. cells in

TABLE VI  
*Inhibition of Various Amounts of Penicillin*

	Cells	Cells + 2000 u. pen./ml.	Cells + 1200 u. pen./ml.	Cells + 800 u. pen./ml.	Cells + 400 u. pen./ml.	Cells + inacti- vated pen. 1600 u./ml.
O <sub>2</sub> -uptake $\mu$ l.	1800	212	254	316	400	1825
Per cent inhibition	—	88	86	83	78	0

Total volume 2 ml. containing 40 mg. lyophilized cells previously incubated in phosphate buffer. 0.25 mM PO<sub>4</sub> buffer, pH 7.0. Time, 6 hrs. Temperature, 30°C.

a total volume of 2 ml. With the range of 400 to 2000 u. crystalline penicillin G/ml. there was little difference in the percentages of inhibition of the oxygen-uptake by the endogenous nucleic acids. Significant inhibition was obtained with amounts of penicillin smaller than 400 u./ml. Because of the lability of penicillin over the period of the experiment, small concentrations gave erratic results. The effect of penicillin inactivated by autoclaving does not inhibit (Table VI).

### DISCUSSION

Since the data reported here reflect the results of an overall metabolic process, *i.e.*, dissimilation of nucleic acid, it is difficult to state whether the effect of penicillin is exerted directly on the metabolic processes of nucleic acid or is the result of some indirect action on these acids. In view of the complexity of the nucleic acids, the metabolic steps involved in their synthesis must be numerous and complex. The site of action of penicillin on these metabolic processes remains to be determined. Comparison of the curves of the endogenous oxygen-uptake with those of sodium ribonucleic acid oxygen-uptake indicates that the effect of penicillin is specific on some metabolic process of nucleic acid metabolism.

The possibility that during the long lag-phase cellular autolysis occurs and, as a result, the subsequent growth of new cells carry out the oxidations, cannot be excluded. Several observations, however, do not support this possibility, particularly those experiments in which the cells have been previously incubated until they have reached their maximum activity before penicillin was added. As mentioned previously, there will be little immediate inhibition but later a definite inhibition will occur. The control experiment with no penicillin exhibited no decrease in oxygen-uptake. Analysis for ribonucleic acid at the completion of such an experiment shows a greater decrease of the ribonucleic content in the absence of penicillin than in its presence. It seems probable that, after maximum activity has been attained, if newly formed cells were responsible for the activity, the number of cells at that time would be the same since the conditions are identical, and that penicillin added at that time would have no effect on the oxygen-uptake, which is contrary to our findings.

Cavalitto *et al.* (13) ascribe the effect of penicillin to its deleterious action on certain SH compounds and enzymes. In our initial survey on

the effect of large amounts of penicillin on various enzyme systems, we tested such enzymes as the triose and succinic acid dehydrogenases which are known to be SH proteins. No effect of penicillin on these systems could be demonstrated. The possibility that a specific SH protein is being inhibited in the chain of nucleic acid metabolism cannot be overlooked; however, it is difficult to explain specificity on such a basis.

The purine nucleotides did not show any activity greater than the endogenous, although the pyrimidine nucleotides possessed some activity which was inhibited by penicillin. It is not known whether these nucleotides have a metabolic function comparable to adenylic acid but, from a structural standpoint, it is likely that they do. Penicillin may be interfering with one of these processes.

Some commercial lots of nucleic acid failed to be oxidized at an appreciably greater rate than shown by the endogenous metabolism. Such samples invariably appeared to be in a higher degree of polymerization than those samples which possessed great activity. From these polymerized nucleic acids a preparation was obtained by hydrolysis with  $\text{NH}_4\text{OH}$  or  $\text{H}_2\text{SO}_4$  (12), which possessed activity greater than the endogenous oxygen-uptake and was inhibited by penicillin. The hydrolysis by  $\text{H}_2\text{SO}_4$  destroys the purine nucleotides but the pyrimidine nucleotides are left intact. The activity of these preparations agrees with those experiments cited above with the isolated pyrimidine nucleotides.

Objection may be raised to the quantity of penicillin required to bring about the inhibition reported. It must be remembered that the quantity of cells (20 mg. dry weight/ml.) is many times in excess of that used when inhibition of growth is obtained by 0.05 u. penicillin/ml. in serial dilution experiments.

#### ACKNOWLEDGMENT

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#### SUMMARY

Penicillin inhibits the dissimilation of the cellular ribonucleic acid and sodium ribonucleic acid employed as a substrate for *Staph. aureus* and other bacteria. The effect apparently is specific in that the dissimilation of a variety of other substrates is not affected by the antibiotic.

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# Studies on Glutamic-Aspartic Acid Transaminase

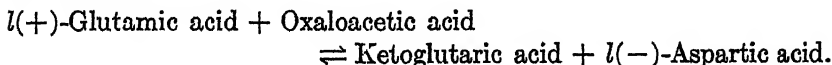
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## INTRODUCTION

The discovery of pyridoxal and pyridoxamine by E. E. Snell (1) and the demonstration of their participation in chemical transaminations (2) of the type first described by Herbst (3) suggested a possible role of these two compounds in biological transamination discovered by Braunshtein and Kritsman (4). In 1944 the authors started to investigate this problem in collaboration with Dr. Snell, and the progress since has been reported at intervals (5, 6, 7). It was demonstrated first that tissues of vitamin B<sub>6</sub>-deficient animals showed a greatly lowered rate of enzymatic transamination (5). In most instances the low rate of activity could be restored to normal *in vitro* by addition of pyridoxal or pyridoxamine in the presence of adenosine triphosphate. Moreover, crude preparations of transaminase obtained by the method of Kritsman (8) as modified by Cohen (9) contained vitamin B<sub>6</sub>. Thus, it became clear that a derivative of vitamin B<sub>6</sub> is essential for and intimately linked with enzymatic transamination. Ultimate proof, however, rested in the isolation<sup>†</sup> and study of the enzyme in highly purified form. Of the two enzymes described by Cohen (9) we selected glutamic-aspartic transaminase for this purpose. This enzyme catalyzes the reaction:



It was found to contain a derivative of vitamin B<sub>6</sub>.

While this work was under way, D. E. Green and coworkers (10) purified both glutamic-aspartic and alanine-glutamic transaminase. Their failure to detect any B complex vitamins in these preparations was revised in a later publication; the role of vitamin B<sub>6</sub> in transamination was admitted (11). Cohen and Lichstein, stimulated

by our first report, examined vitamin B<sub>6</sub>-deficient microorganisms (*S. fecalis* R) for lowered transaminating power. They contested our claim for the role of vitamin B<sub>6</sub> (12), but this opinion was revised in a later report by Lichstein and others (13). The investigations of Gunsalus and his group on bacterial transaminase have been particularly successful in establishing the role of vitamin B<sub>6</sub> (14).

In this paper the preparation of glutamic-aspartic transaminase from mammalian tissue and some of its properties are described. The product obtained is almost twice as active as that described by others (11), and it may represent the pure compound. Although large losses of active material occur, the yield is many times higher than that obtained by earlier isolation procedures (11), while the amount of source material and the equipment needed for the isolation are moderate.

## EXPERIMENTAL PART

### *Analytical Procedures*

Since Braunshtein and Kritsman's early communications, the development of new analytical techniques by P. P. Cohen (9) has greatly facilitated the study of transaminases. His chloramine method was used throughout this investigation. As a check, and for comparison, we have recently employed the method of Ostern (15) as modified by Green (11). The result of purification is expressed by the  $Q_{\text{Transaminase}}$  value: microliters substrate transaminated/hr./mg. of enzyme (dry weight) (16). For details the original papers may be consulted.

The  $Q_T$  values given below pertain to the experimental conditions given by Cohen (9).  $Q_T$  values obtained by the technique of Green and coworkers (11) are 1.1 times higher.

The protein values are based on micro Kjeldahl determinations. Ammonium sulfate was removed from the solutions by dialysis before the analytical procedures.

### *Isolation of the Enzyme*

In accordance with Kritsman (8), pig heart was found to be the most convenient source. Pig liver and beef heart are less suitable. The following purification steps were elaborated:

1. *Dehydration by Acetone.* The hearts may be stored in the frozen state or may be worked up immediately after slaughtering. The material is ground in a meat grinder (coarse and fine) and the resulting pulp is stirred into 5 volumes of ice-cold high grade acetone. After 10 minutes the mixture is filtered through several layers of gauze. If fine particles escape into the filtrate, they may be recovered by subsequent filtration through soft paper in a Buchner funnel. The combined residues are stirred up again in cold acetone (5 volumes) and filtered as before. The material is dried as rapidly as possible on filter paper in large vacuum desiccators. The acetone-treated dry material retains its activity for several weeks when stored dry and cold. From 1 kg. of fresh pig heart ( $Q_T = 600-700$ ) 200 g. of dry product are obtained.

2. *Extraction.* Beginning with this step, the solutions containing the enzyme should not be exposed to bright daylight for long periods (7). The inactivation by diffuse daylight as encountered in average laboratory rooms is negligible.

Fifty g. of acetone-treated heart are extracted with 350 ml. of 0.04  $M$   $\text{Na}_2\text{HPO}_4$  solution at 55–60°C. for 30 minutes. The phosphate solution should be prewarmed. Filtration through a fluted filter and subsequent manual pressing of the filter in a gauze bag yields 300 ml. of cloudy extract (pH 6.5 to 7), containing 4–5 g. of protein ( $Q_T = 1500$ ).

3. *Removal of Inert Proteins by Isoelectric Precipitation.* The extract is cooled in an ice bath and 1  $N$  acetic acid to pH 4.5 is added with vigorous mechanical stirring (requires about 6 ml. of acid/100 ml. solution). The resulting precipitate is removed immediately by centrifugation. The solution is reddish brown and clear. Under cooling and stirring the pH is adjusted to 7.5, using approximately 7 ml. of 1  $N$   $\text{NaOH}$  for each 100 ml. The acidification is the only phase in the course of isolation in which particular care has to be exercised and no interruption should occur. About 300 ml. of clear solution ( $Q_T = 3000$ ) are obtained. The protein content is 1.5–2.0 g.

4. *First Ammonium Sulfate Precipitation.* For this a nearly saturated solution of ammonium sulfate containing some ammonia is used. We found the following mixture convenient: 487 g. of ammonium sulfate and 1.5 ml. of conc. ammonia solution ( $d = 0.9$ ; 28–29%  $\text{NH}_3$ ) are dissolved with water to 1 liter; the pH of the mixture on dilution is about 7.5 as measured with the glass electrode (Beckman pH meter). The precipitation of the protein is carried out below room temperature under mechanical stirring. A separatory funnel or a pipette with a narrow tip is used. The outlet should be below the surface of the liquid. For each 100 ml. of solution, 100 ml. of ammonium sulfate solution are added. The inactive precipitate is centrifuged down or filtered off, using a hard filter paper. The first portions should be refiltered if more than a trace of cloudiness is seen. To the filtrate another 165 ml. of ammonium sulfate solution per 100 ml. original solution are added with the precautions outlined above. The resulting precipitate contains the enzyme. It is separated from the solution by filtering through hard grade filter paper, refiltering if necessary. When the filtration begins to slow, an automatic device can be used to keep the level of the liquid on the filter constant. We found it convenient to put the solution into an inverted bottle with a narrow spout extending to the desired level in the funnel. The filtration is usually finished over night. It should be carried out in an icebox. 700 mg. of protein are obtained in this step ( $Q_T = 5000$ ).

5. *Second Ammonium Sulfate Precipitation.* The filter paper is removed and spread out on a large watch-glass. The enzyme is dissolved with distilled water in several 5 ml. charges, carefully soaking all parts of the filter. The extracts are combined in a previously weighed 25 ml. volumetric flask and are diluted to the mark. Weighing the solution gives approximate information (17) about the concentration of ammonium sulfate from the filter, and will facilitate subsequent fractionation.

Example: 25 ml. solution at 20°C. weighed 26.24 g.;  $d = 1.0495$ . An ammonium sulfate solution of this density contains 90 mg. of the salt/ml. (17).

The protein content of the solution can be disregarded in the approximate calculation. For the fractional precipitation, the same precautions used in the first ammonium sulfate fractionation should be observed. Three or four fractions should be obtained.

Example: 25 ml. of transaminase solution containing 90 mg. ammonium sulfate/ml. (see above) were used. The salt concentration was increased to 245 mg./ml. by adding 16 ml. of the ammonium sulfate solution specified in 4. Fractions 2, 3 and 4 were



obtained by successive addition of 8 ml., 8 ml. and 20 ml. of ammonium sulfate solution. The concentrations were then 283 mg., 311 mg. and 355 mg./ml. solution.

Each precipitate is filtered off, dissolved in 20 ml. 0.01 *M* phosphate buffer (pH 7.5), and dialyzed in cellophane tubing for 24 hours against three charges of 0.01 *M* phosphate buffer, pH 7.5. The activity of each fraction should be determined, and the most active fractions are used for further purification. An example for the course of a fractionation, the yield and the activity is given in Table I.

TABLE I  
*Second Ammonium Sulfate Fractionation of Glutamic-Aspartic Transaminase*

Fraction No.	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Concentration	Protein precipitated	Purity: <i>Q<sub>T</sub></i> value
	mg./ml.	mg.	
1	245	63	4100
2	283	126	8200
3	311	110	9400
4	355	90	6300

6. *Purification with Alumina.* The solution is diluted to a volume of 50 ml. and brought to pH 5.8 by addition of a few drops of 0.1 *M* acetic acid. Under vigorous mechanical stirring, alumina C<sub>γ</sub> suspension (18) is added in fractions. This suspension should have a dry weight of 10 mg. Al<sub>2</sub>O<sub>3</sub>/ml. If other concentrations are used, the amounts given below should be adjusted. The first fraction (a) is obtained by dropwise addition of 5 ml. of the alumina C<sub>γ</sub> suspension. It is centrifuged. The second fraction (b) is obtained by adding 10 ml. alumina C<sub>γ</sub> suspension to the supernatant of fraction a. Centrifuge. A third fraction (c) is obtained by adding 75 ml. alumina C<sub>γ</sub> suspension to the supernatant of fraction b. Centrifuge. The supernatant from fraction c may be tested for unadsorbed enzyme. The precipitates are each washed with 100 ml. cold distilled water and, after centrifugation, the enzyme is eluted from the alumina with 50 ml. 0.04 *M* phosphate buffer, pH 7.5. The alumina should be brought into suspension, stirred carefully and shaken occasionally for an hour. By centrifugation, water-clear solutions of the enzyme are obtained. The alumina from which the most active fraction is obtained may be re-extracted with buffer. A second eluate of lower activity is obtained. The activity and protein content of each fraction are determined. Depending on the contaminations present in the ammonium sulfate fraction used, the purest enzyme is found in either the second or the third fraction. An example of this purification step is given in Table II. Fractions 2 and 3 of the preceding step were used for this.

It is not yet possible to state whether the product with a *Q<sub>T</sub>* of 25,000 to 26,000 represents the pure enzyme, but we are inclined to assume this, since, despite many attempts, we have so far been unable to obtain more active preparations by repeating and modifying our purification steps. Preparations with a *Q<sub>T</sub>* = 25,000 measured ac-

TABLE II  
*Fractional Adsorption of Glutamic-Aspartic Transaminase on Alumina*

Fraction No.	Mg. of protein eluted	Purity: $Q_T$ value
2a	28.6	2,300
2b	14.8	16,500
2c	18.0	26,500
3a	10.0	3,000
3b	26.0	10,500
3c	12.0	24,500

cording to Cohen's procedure (19) have a  $Q_T = 28,000$  in the test system described by Green and coworkers (11). This value is nearly twice as high as that reported by the latter authors for their preparation. The discrepancy remains to be explained. It may be that some decay of the enzyme (20) occurred in the course of the preparation.

The concentration in the source material is surprisingly high. The losses during the purification are considerable, but the material used is inexpensive.

#### SOME PROPERTIES OF GLUTAMIC-ASPARTIC TRANSAMINASE

*Stability.* The purified enzyme is stable in solution when stored in an ice box. No significant loss of activity is observed within 2 weeks. After 4 months storage without special precautions, we found the activity decreased by one-third. Exposure to oxygen does not inactivate the preparations. Heating to 80°C. and above will destroy the enzyme within a few minutes, but heating to 60°C. for as long as one hour does not impair it. The enzyme is rather stable in neutral medium and even at pH 9 to 10, but is inactivated within a short time in an acid medium below pH 4.5. It can be obtained in dry and stable form by evaporation in the frozen state.

*Inhibitors.* In accordance with previous investigators (21, 22) we found no specific inhibitors active in low concentration. An inhibition of 50% is observed with  $0.5 \times 10^{-6} N$   $Ag^+$ , and with  $1 \times 10^{-6} N$   $Hg^{++}$ . Typical aldehyde reagents, such as hydroxylamine, semicarbazide and dihydromethylresorcinol, inhibit only at much higher concentration even if mixed with the enzyme prior to the addition of substrate (keto acid).

We were able to confirm the data on specificity, equilibrium of the reaction, and pH optimum for activity reported by Cohen for the crude enzyme (16).

#### PROSTHETIC GROUP

The demonstration of the presence of a derivative of vitamin B<sub>6</sub> was complicated by the fact that the prosthetic group is very firmly bound to the protein. The usual measures for the removal of coenzymes, such as prolonged dialysis over a pH range of 6 to 10, and attempts to reactivate by concentrated dialyzate or decoctions of tissues and by various vitamin B<sub>6</sub> derivatives, proved ineffective. The presence of a distinct prosthetic group, however, was strongly suggested by several observations in addition to our experiments with deficient tissues. Using homogenized tissues from vitamin B<sub>6</sub>-deficient rats, a restoration of transaminase activity was accomplished by the addition of pyridoxal or pyridoxamine and adenosine triphosphate, but not by pyridoxine and adenosine triphosphate, or adenosine triphosphate alone (5). An increase in vitamin B<sub>6</sub> content was noted on purification of the enzyme.\* The light sensitivity of transaminase was found to parallel that of pyridoxine and its derivatives (7).

The demonstration of Gunsalus and others that tyrosine codecarboxylase (23) is identical with pyridoxal phosphate (24, 25, 26) suggested to us that the prosthetic group of transaminase may be a phosphorylated derivative of vitamin B<sub>6</sub>. After prolonged dialysis against distilled water, our preparations retain organic phosphate in an amount amply sufficient to justify this assumption. We finally resorted to chemical determinations using previously known colorimetric reactions. For this, the prosthetic group has to be removed from the protein, the phosphate group has to be hydrolyzed, and the protein has to be removed quantitatively to avoid interference. Both the 2,6-dichloroquinone chloroimide reagent (27, 28) and diazotized sulfanilic acid can be used (29). With the latter reagent pyridoxal gives a lemon yellow color, while pyridoxine and pyridoxamine give an orange to pink color (30). Pyridoxine is not likely to play a role as prosthetic group, since it does not activate *in vitro* transamination with homogenized deficient tissues.

Our method of isolation usually yields preparations with pyridoxal as component of the prosthetic group. In a few instances, however, the

\* The authors are indebted to Dr. E. E. Snell for microbiological assays.

pyridoxamine color was obtained with diazotized sulfanilic acid. In which state the prosthetic group is found may depend on the source material. Earlier we had reported the occurrence of both pyridoxal and pyridoxamine, as determined by microbiological assay (7), in one and the same transaminase preparation. It would seem probable that pyridoxal transaminase and pyridoxamine transaminase represent the two fractions of equal activity but different electrophoretic mobility as reported by Green (11).

Our findings corroborate that both pyridoxal and pyridoxamine may be part of the prosthetic group. Indeed, the assumption (E. E. Snell) that the capacity for transamination is due to the reaction:



has been the initial impulse for this investigation. Conclusive proof for this mechanism was furnished recently by Umbreit, O'Kane and Gunsalus (14).

Attempts to determine the amount of prosthetic group in transaminase are beset with difficulties and the accuracy should not be overrated. We have used several methods described by others, and combinations thereof. For quantitative liberation of the phosphate group, 3 hours hydrolysis in 1 *N* HCl or H<sub>2</sub>SO<sub>4</sub> was carried out (31). Control experiments with pyridoxal and pyridoxamine showed that the destruction under these conditions amounts to less than 10%. Similarly, the adsorption and elution procedures were checked for recovery; it is almost quantitative. The final volume of the samples was kept small, and the amounts of the reagents prescribed were diminished in proportion.

Micro cuvettes were used for some of the measurements. Examples:

1. A sample of 4.65 mg. transaminase ( $Q_T = 23,500$ ) was hydrolyzed, adsorbed on Lloyd's reagent, eluted and tested with diazotized sulfanilic acid. Found: 18 $\gamma$  of pyridoxal, 0.46%.

2. Two samples (*a* and *b*) of transaminase ( $Q_T = 20,000$ ), 3.8 mg. of protein in each, were hydrolyzed and deproteinized by trichloroacetic acid. Sample *a* was irradiated with ultraviolet light to destroy vitamin B<sub>6</sub> compounds (7). Determination with diazotized sulfanilic acid; value *a* was subtracted from value *b*; found: 7.2  $\gamma$  of pyridoxal, 0.19%.

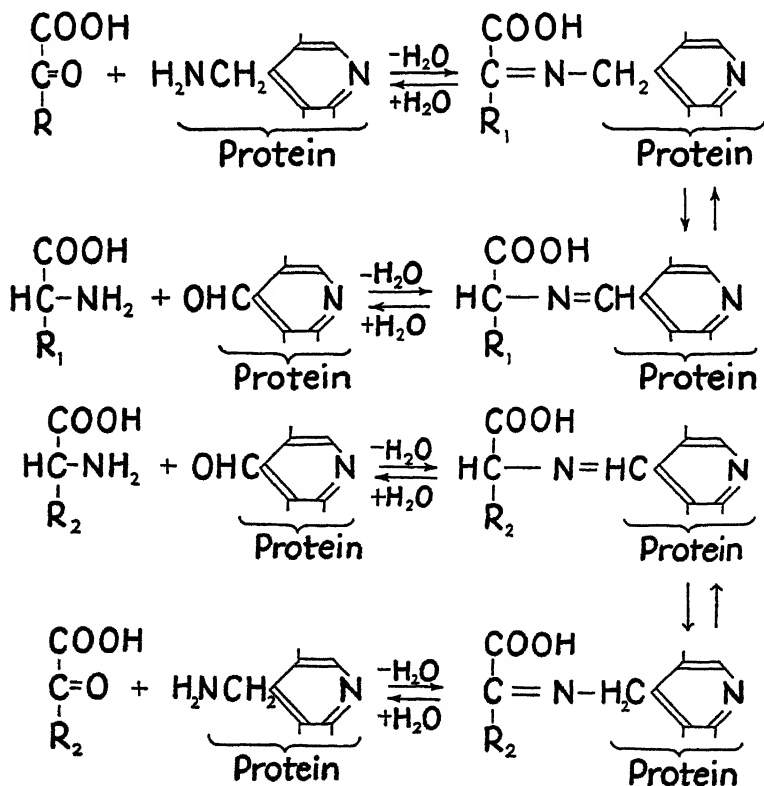
3. A sample of 17.8 mg. transaminase ( $Q_T = 25,000$ ) was hydrolyzed, adsorbed on Lloyd's reagent, eluted: 37.2  $\gamma$  of pyridoxamine, 0.21%.

4. A sample of 14.3 mg. transaminase ( $Q_T = 26,000$ ) was hydrolyzed in 1 *N* H<sub>2</sub>SO<sub>4</sub>; the acid was removed with Ba(OH)<sub>2</sub> and the solution was evaporated. The residue was extracted with alcohol and the vitamin B<sub>6</sub> content was determined with diazotized sulfanilic acid; 33.3  $\gamma$  of pyridoxal, 0.23%.

In other tests superfiltrol (32) and zeolite (33) were used for adsorption and, after the hydrolysis, deproteinizing with tungstic acid was carried out. In some determinations the dichloroquinone chloroimide reagent (34) was used. The values obtained ranged between 0.1 and 0.5% pyridoxal or pyridoxamine.

### DISCUSSION OF RESULTS

The concentration of glutamic-aspartic transaminase in heart muscle seems to be surprisingly high (about 2%). If sufficient substrate were available and no regulators were present, heart muscle would metabolize two or three times its own weight of substrates per hour. This illustrates that transaminase is a powerful enzyme which rapidly interconverts key substances of carbohydrate and protein metabolism.



The recognition of the role of vitamin B<sub>6</sub> derivatives in this process permits the modification and extension of the equations proposed by Braunshtein and Kritsman (4) for an explanation of the mechanism of transamination. The scheme of Karrer (35), which requires an auxiliary enzyme for intermediary hydrogenation and dehydrogenation of the Schiff base, may be disregarded, since we now know that the process proceeds with highly purified and perhaps uniform preparations. The formulas given on page 76 resemble Braunshtein and Kritsman's original scheme, but pyridoxal and pyridoxamine are forming the Schiff base instead of direct combination between keto and amino acid. For simplicity the substituents of the pyridine base have been omitted in the formulae.

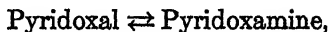
One of the functions of another vitamin as prosthetic group of an enzyme seems to be settled by this and concurrent investigations (5, 11, 13, 14). The remarkable feature of it is that the function was predicted from the chemical configuration by E. E. Snell (1, 2). The substrate specificity, however, and the high speed with which the reversible combination between enzyme and substrate takes place remain the mystery of the protein moiety of the enzyme.

### SUMMARY

1. A method for the preparation of glutamic-aspartic acid transaminase from pig heart is described.

2. Experiments to identify and estimate the prosthetic group of this enzyme are reported. Pyridoxal phosphate is present in most instances in the purified preparation but, in a few instances, a pyridoxamine derivative was found.

3. The enzyme most probably acts by the change:



in the prosthetic group.

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# Note on the Colorimetric Determination of Pyridoxine, Pyridoxal and Pyridoxamine

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## INTRODUCTION

In the course of our work with glutamic-aspartic transaminase (1) it became necessary to determine derivatives of vitamin B<sub>6</sub>, particularly pyridoxal and pyridoxamine. Since microbiological assays are difficult for several reasons (2, 3), we tried to apply some of the chemical methods described for the determination of pyridoxine. Most of the reagents recommended for this purpose give the same color with all vitamin B<sub>6</sub> derivatives, although there are differences in intensity and stability.

It was found, however, that, by the use of diazotized sulfanilic acid, it is possible to distinguish between pyridoxal, pyridoxamine and pyridoxine. This reagent was first considered for the determination of vitamin B<sub>6</sub> by Kuhn and Löw (4). Swaminathan (5) and, later, Bina, Thomas and Brown (6) used it for the quantitative determination of pyridoxine. It may be mentioned that the *p*-aminoacetophenone reagent recommended later (7) is not as suitable for pyridoxal and pyridoxamine as it is for pyridoxine.

Using diazotized sulfanilic acid, an orange color is obtained with pyridoxine, an orange to pink color with pyridoxamine, and a bright yellow color with pyridoxal.\* The absorption spectra are shown in Fig. 1.

The stability of the colors obtained is very limited. In each case the maximum intensity is reached one minute after addition of the diazotized sulfanilic acid. The pyridoxamine color begins to fade

\* The authors are indebted to Dr. R. T. Major, Merck and Co., for a generous gift of pyridoxal and pyridoxamine.



rapidly after 3 minutes, while the absorption maximum of the pyridoxine product begins to shift toward shorter wave lengths. The pyridoxal color is stable for about 5 minutes.

In Fig. 1 the absorption spectrum of the dye obtained in the same fashion from the dephosphorylated prosthetic group of a transaminase

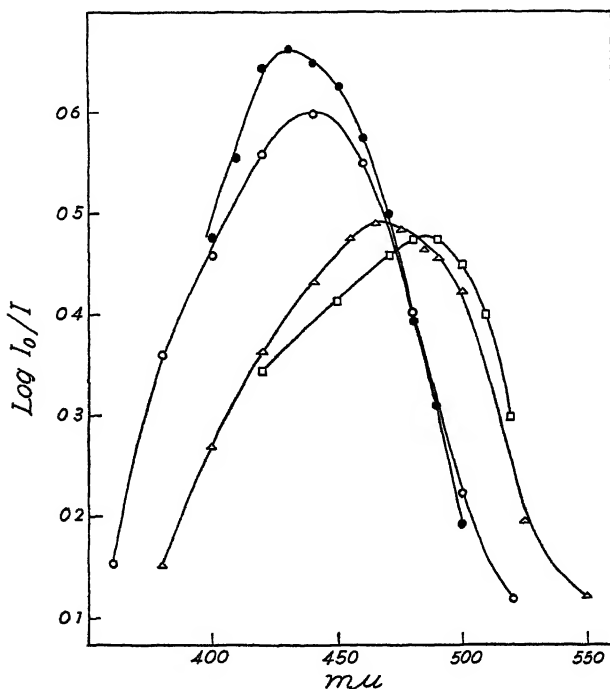


FIG. 1. Absorption Spectra of Dyes Obtained from Vitamin B<sub>6</sub> Derivatives with Diazotized Sulfanilic Acid.

Pyridoxine: Δ—Δ—Δ

Pyridoxal: ○—○—○

Pyridoxamine: □—□—□

Prosthetic group of transaminase: ●—●—●

preparation is also shown. It resembles the absorption of the pyridoxal dye and the lability of the color is the same.

This favors the assumption that the prosthetic group of transaminase may be closely related to pyridoxal. Other experiments indicating the relationship between vitamin B<sub>6</sub> compounds and the prosthetic group of transaminase have been reported in the preceding paper (1).

## EXPERIMENTAL PROCEDURES

The reagents used were those described by Bina, Thomas and Brown (6). In most instances we used one-half or one-fourth the volumes specified by these authors. The absorption spectra represented in Fig. 1 were taken with a Cenco-Sheard spectrophotometer. The light path was 2 cm., the concentration 0.2  $\gamma$  moles of each compound in 6 ml. of H<sub>2</sub>O. The transaminase was prepared according to the method given in the preceding paper (1). The purity was Q<sub>T</sub> 23,000. Of this preparation 13.3 mg. were hydrolyzed in 1 N H<sub>2</sub>SO<sub>4</sub> for 3 hours (8) to liberate the prosthetic group from the protein and to split off the phosphate group. The vitamin B<sub>6</sub> derivative was adsorbed on Lloyd's reagent and eluted by 0.01 N NaOH. The solution was neutralized prior to adding the reagents. Assuming that pyridoxal is the B<sub>6</sub> compound present in this transaminase preparation, the content of the sample can be calculated approximately from the absorption value (see Fig. 1). It is 36.8  $\gamma$  or 0.28%.

## SUMMARY

1. Pyridoxal, pyridoxamine and pyridoxine give different colors when treated with diazotized sulfanilic acid.

2. An application of the test to the examination of the prosthetic group of a preparation of glutamic-aspartic transaminase is reported.

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# A Comparative Study of the Blood and Liver Catalases from the Horse

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## INTRODUCTION

Since Sumner and Dounce (1) succeeded in crystallizing beef liver catalase in 1937 a great many methods have been devised to produce pure catalase from different sources.

The activity of the catalase preparations made by Sumner and his coworkers (1) varied considerably, horse liver catalase, *e.g.*, from Kat.f. 22,000 to 50,000.

Agner (2) obtained crystalline liver catalase from horse (1942). His preparations differ from those of Sumner in having a greater, and constant, activity of about 60,000. The iron content, about 0.09%, agrees with that of Sumner's preparations.

In 1943 Agner (3) prepared catalase from horse erythrocytes. The iron content was 0.087%. In 1944 this catalase was crystallized in this institute (not published) with an iron content of 0.093%, hemin content of 1.085% and an activity of 65,000.

The immuno-chemistry of catalase has been studied by Tria (4), who found that beef liver catalase gave a flocculent precipitate with immune rabbit serum. Lamb liver catalase also gave a precipitate, while horse liver catalase gave only a slight precipitate.

At the same time, Campbell and Fourt (5) showed that the horse liver catalase titer against immune rabbit serum was much less than that of beef liver catalase. Also, dog liver catalase gave a precipitate, but neither hematin nor beef hemoglobin gave any reaction.

The nitrogen distribution and the basic amino acids have been determined on pure horse liver catalase by Theorell and Åkeson (6) by means of a new micro method.

From earlier publications of various authors (Sumner (1), Lemberg (7), Agner (2) ) and from the analyses described later in this paper it must be assumed that horse blood catalase has 4 hemin groups and horse liver catalase has 3 hemin and 1 "verdohemochromogen" group per molecule.

It now seemed of interest to ascertain whether this difference in the number of hemin groups was accompanied by any difference in the protein component, or if the only difference was that of the hemins.

This has been investigated by means of amino acid analyses, ultra-violet absorption measurements and precipitin reactions.

## EXPERIMENTAL

### *Preparations*

*Preparation of Crystalline Horse Blood Catalase.* 3.5 l. of blood corpuscles were washed three times with 0.9% NaCl and hemolysed by adding 7 l. of distilled water.

The total amount of catalase was, according to activity measurements, calculated to be 1.4 g. with an activity of Kat.f. = 65,000.

3 liters of a 96% alcohol-chloroform mixture (3:1) were added with vigorous stirring. After 12 hours the solution was filtered. The filtrate showed a clearly visible hemoglobin spectrum.

The clear yellow red solution was evaporated *in vacuo* to 1 liter. This still contained hemoglobin and methemoglobin. A distinct catalase band at  $625\text{ m}\mu$  was, however, now visible after reducing a small sample with  $\text{Na}_2\text{S}_2\text{O}_4$  in addition to those of hemoglobin.

The solution was made 0.1 *M* with respect to acetate buffer, pH 4.00 and, in the course of a few hours, a large greyish-brown precipitate of denaturated protein settled and the hemoglobin spectrum disappeared completely.

After centrifuging, 680 ml. of acetone was added to 1 liter of solution. A large amount of greyish, denaturated protein was precipitated and centrifuged. A further amount of 320 ml. acetone was added to the clear solution whereupon the catalase, strongly colored green by the acetate (Agner and Theorell 8), was precipitated together with some denaturated protein. The mother liquor was yellow, but did not show any catalase spectrum.

The catalase was dissolved in 200 ml. 0.1 *M* acetate buffer, pH 4.00, and centrifuged clear.

200 ml. 96% alcohol was added and a greyish-brown precipitate was centrifuged and discarded. A further amount of 250 ml. of alcohol was added and the still green colored catalase was precipitated and dissolved in 25 ml. acetate buffer as before.

The solution was dialyzed against distilled water and a small amount of white, denaturated protein was removed. A saturated solution of ammonium sulphate was now added until the catalase began to precipitate at an ammonium sulphate saturation of 0.45. The catalase then crystallized without further addition of ammonium sulphate. The crystals had the shape of small needles which were easily dissolved in distilled water. The yield was 450 mg. of crystalline catalase.

If the catalase does not crystallize as described, the reason is probably that the solution had not been standing long enough with acetate buffer. In this case the catalase is precipitated in the amorphous state by adding more ammonium sulphate and redissolved in acetate buffer. In a few hours the rest of the greyish-brown protein

will precipitate out and the catalase can be crystallized as described, without being dialyzed again.

*Crystallization from Water.* A dialyzed solution of 1% catalase in water was electro-dialyzed. During the dialysis it began to crystallize out and after a few hours in an ice-chest most of the catalase had crystallized as prisms, see Fig. 1. The crystals were easily dissolved in  $N/1000$  ammonia.

When the catalase in dialyzed water-solution is kept in an ice-chest it usually crystallizes in 2-3 weeks. Once crystallized from water it is necessary to keep it in  $N/1000$  ammonia or it may crystallize from day to day.

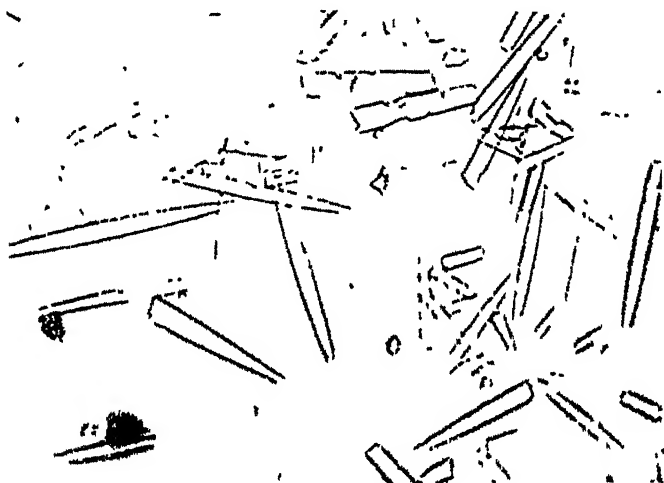


FIG. 1

Crystalline Horse Blood Catalase (from Water Solution) 200  $\times$ .

An attempt was made to crystallize the catalase in the presence of acetate. A 0.9% solution was made 0.2  $M$  with respect to acetate buffer, pH 4.00, and in a couple of days 20% crystallized out as thin, rhomboid plates, see Fig. 2. In acetate buffer 0.2  $M$ , pH 4.00, the acetate is bound to the hemin iron (Agner and Theorell (8)) and, from the difference in form of the crystals and the solubility in water, we assume that the crystals consist of catalase-acetate.

*Preparation of Crystalline Horse Liver Catalase.* The first and second steps were carried out as described by Agner (2). The solution was then made 0.1  $M$  with regard to acetate buffer, pH 3.8. In the course of a few hours a large greyish-brown precipitate of denatured protein appeared, and was centrifuged off.

A fractionation with alcohol was carried out, according to Agner's step 4 and the solution was dialyzed against distilled water. A small precipitate of brown denatured protein was centrifuged off and discarded.

The catalase was now crystallized from ammonium sulphate as described by Agner.

If the crystallization is not successful the same procedure as used for the blood catalase must be followed.

When placed in an ice-chest in dialyzed water solutions the liver catalase crystallizes out in long rods; 7 mm. rods have been obtained.

*Preparation of Crystalline Human Blood Catalase.* 5 l. of blood corpuscles were freed from serum by means of a Laval cream separator and hemolyzed by adding double the amount of distilled water. The amount of catalase present was, according to activity measurements, calculated to be 2.5 g. with an activity of Kat.f. 50,000.

7.5 liters of a 96% alcohol-chloroform mixture (3:1) were added with vigorous stirring. The large amount of protein precipitate absorbs much of the alcohol-chloroform, which must be squeezed out and the alcohol-chloroform thus obtained added to the solution. The stirring must continue for several minutes.

After about an hour the precipitate can, in most cases, be filtered off. If this is not the case the stirring must be continued, if necessary with the addition of a further small amount of alcohol-chloroform.



FIG. 2

Crystalline Horse Blood Acetate-Catalase (from Acetate Buffer) 210  $\times$ .

The filtered solution should be clear with a very faint yellow-green color without any hemoglobin spectrum.

It was then evaporated at reduced pressure to 500 ml.

310 ml. of saturated ammonium sulphate solution was added. A greyish-brown precipitate was centrifuged out and discarded. A further amount of 190 ml. ammonium sulphate solution was added, whereupon the catalase settled as a grey precipitate. The mother liquor was yellow. The solution was then fractionated with acetone and alcohol according to the procedure previously described for the preparation of horse blood catalase, the fractionation in this case being carried out at 0°C.

The solution was then dialyzed against distilled water and a small white precipitate of denatured protein removed.

Saturated ammonium sulphate was added to a saturation of 0.41, when a faint turbidity appeared which was removed by centrifuging. The degree of saturation was then increased to about 0.43, when the catalase crystallized out. On repeating the same process no initial turbidity appeared and crystallization starts immediately.

The crystals had the form of small needles and were dissolved in distilled water. The yield was 280 mg.

After electrodialysis of the dialyzed solution, in a few days 100 mg. crystallized in the form of short prisms, see Fig. 3.

As the catalase was used for other analyses there was not enough material left for carrying out amino acid analyses, but enough remained for the precipitin reactions.



FIG. 3

Crystalline Human Blood Catalase (from Water Solution) 210  $\times$ .

### Analyses

The hemin content was determined as pyridine-hemochromogen, the iron as described by Agner (2). The nitrogen content by means of micro Kjeldahl. Kat.f. was determined according to v. Euler and Josephson (9): 0.25 to 0.30  $\gamma$  was taken for each determination,  $k$ , determined by extrapolation. It must be added that we found the accuracy of the activity determination to be about  $\pm 5\%$ .

TABLE I

Catalase from	Kat. f.	Hemin	Nitrogen	Iron
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Horse liver	50,000	0.91-0.89	16.8	0.095-0.091
Horse blood	65,000	1.090-1.070	16.8	0.095-0.091
Human blood	50,000	0.83	16.7	0.077

No further control of purity was carried out on the human blood catalase samples but a fairly high degree of purity can be assumed judging by the aspect of the crystals. It is remarkable that human blood catalase has such a low iron and hemin content. If we assume the same molecular weight as for the other catalases, this would mean that this catalase contains only 3 hemin groups. No biliverdin was formed on



addition of acetone-HCl as earlier stated for the beef and horse blood catalase by Laskowski and Sumner (10) and by Agner (3).

Electrophoretically, in a Tiselius apparatus at pH 3.9 in acetate buffer, and pH 7.5 in phosphate buffer, and migration through  $\frac{3}{4}$  of a cell, horse liver and blood catalase showed complete homogeneity.

### *Amino Acid Analyses*

For the determination of the nitrogen distribution and the separation of the amino acids as basic, neutral and acid, the method of Theorell and Åkeson (6) was followed in detail.

The catalase solutions used derived from two different preparations.

The cathode fraction was analyzed for nitrogen, and for histidine according to Jorpes (11), and for arginine according to Jorpes and Thorén (12), while lysine was calculated as difference.

The cathode-N was taken as the mean value of two determinations that did not differ more than 1.5% from each other.

The results were as follows:

TABLE II  
*Horse Blood Catalase*

Fraction	Per cent of total nitrogen			
	Preparation 1			Preparation 2
	Analysis 1	Analysis 2	Analysis 3	Analysis 4
Humin	1.2	1.5	—	1.2
Amide	7.8	9.2	—	9.5
Neutral	41.2	41.0	—	40.0
Anode	16.6	15.4	—	16.9
Cathode	32.3	32.0	32.3	31.6

TABLE III  
*Basic Amino Acids (per cent of Dry Weight) in Horse Blood Catalase*

	Preparation 1			Preparation 2
	Analysis 1	Analysis 2	Analysis 3	Analysis 4
Histidine	4.34	4.15	4.00	4.19
Arginine	8.87	8.87	8.80	8.46
Lysine	7.28	7.29	7.87	7.54

TABLE IV  
*Horse Liver Catalase*

Fraction	Per cent of total nitrogen		
	Preparation 1	Preparation 2	
	Analysis 1	Analysis 2	Analysis 3
Humin	1.8	1.2	—
Amide	8.1	9.0	—
Neutral	40.6	41.2	—
Anode	17.3	16.1	—
Cathode	31.6	31.1	30.8

TABLE V  
*Basic Amino Acids (per cent of Dry Weight) in Horse Liver Catalase*

	Preparation 1	Preparation 2	
	Analysis 1	Analysis 2	Analysis 3
Histidine	3.65	3.96	3.96
Arginine	8.81	9.05	8.85
Lysine	7.74	6.46	6.54

Tyrosine was determined in the neutral fractions. The method described by Thomas (14) was used.

In horse blood catalase we found 6.1% tyrosine (*per cent of dry weight*) in analysis 1 (see Table II), 6.1% in analysis 2 and 5.7% in analysis 4.

In horse liver catalase we found 5.7% tyrosine (*per cent of dry weight*) in analysis 1 (see Table IV), 5.8% in analysis 2 and 5.9% in analysis 3.

Cystine, glutamic and aspartic acid determinations were made according to a chromatographic method described by Darling (13), based on works by Wieland (16), Turba (15) and others.

Samples of 20–30 mg. were used for each determination and were hydrolyzed as described by Theorell and Åkeson (6). The HCl was removed by alternate drying on a water bath and addition of a little water.

The insoluble part of the humin was centrifuged off and the solution made faintly alkaline to phenolphthalein by adding KOH. The solution was diluted to 10 ml. and then passed through the column of alumina.

KOH was added to a pH of about 8 to the fraction which was eluted with 30 ml. of water. It contains the basic and neutral amino-acids, and all of the amide-nitrogen in the form of ammonia. The latter was distilled over into a receiver containing HCl and titrated. The nitrogen content of the remainder was determined.

The glutamic acid was eluted with acetate, the aspartic acid with KOH in the same amounts as used by Darling. The eluates were then separately evaporated over a gas flame and burned in 50 ml. combustion flasks, with 2 ml. of concentrated  $H_2SO_4$  because of the large amount of salt.

A series of blank tests was carried out as described by Darling. They varied very little but gave slightly higher values than those arrived at by Darling. We found 0.015 mg. nitrogen in the aqueous eluate, 0.019 mg. nitrogen in the acetate eluate and 0.018 mg. nitrogen in the basic eluate. The blank tests were subtracted from the results.

Standard determinations were made with known amino acid solutions. Histidine plus alanine gave an error of  $\pm 1.5\%$ , glutamic acid  $\pm 1.0\%$  and aspartic acid  $\pm 2.1\%$ .

Cystine was also adsorbed in the column. In a test experiment 5.9 mg. cystine was quantitatively recovered by elution with acetate.

As it was not known whether cystine was present or not, a direct determination was made on acetate eluates.

The determinations were made according to Kassell and Brand (17). In the "liver" fraction 1.85% of cystine was found calculated on the catalase dry weight and in the "blood" 1.65% (one determination).

The amount of nitrogen corresponding to this cystine value was subtracted from the glutamic acid nitrogen and added to the basic and neutral fraction.

If the cystine content was calculated from the amount of nitrogen exceeding the 16.5% corresponding to the glutamic and aspartic acids, as determined with the electrodialysis apparatus, the content of cystine thus found agreed as well as might be expected with that directly determined.

The results were:

TABLE VI  
*Horse Blood Catalase*

Fraction	Per cent of total nitrogen			
	Analysis 1	Analysis 2	Analysis 3	Analysis 4
Amide	8.4	11.5	11.0	10.0
Neutral and basic	71.3	69.5	71.0	72.5
Glutamic and aspartic	16.2	15.9	16.8	16.9
	Per cent of dry weight			
Glutamic acid	10.0	10.5	11.1	11.9
Aspartic acid	16.8	16.0	16.8	16.2

Cystine, direct determination = 1.65

TABLE VII  
*Horse Liver Catalase*

Fraction	Per cent of total nitrogen			
	Analysis 1	Analysis 2	Analysis 3	Analysis 4
Amide	10.0	9.6	—	83.8
Neutral and basic	70.9	73.2	71.0	
Glutamic and aspartic	16.4	16.1	16.0	16.2
	Per cent of dry weight			
Glutamic acid	10.5	10.6	9.7	10.3
Aspartic acid	16.7	16.1	16.7	16.6

Cystine, direct determination = 1.85

### *Absorption Spectrum*

This determination was made in the apparatus described by Warburg and Negelein (18).  $\beta$  was calculated from a molecular weight of 225,000 for both catalases.

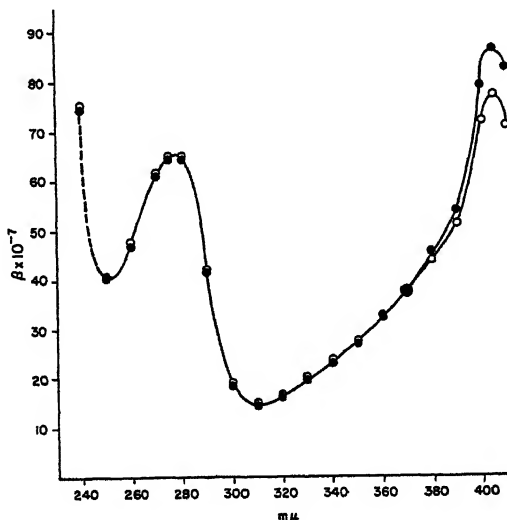


FIG. 4

Phosphate buffer pH 7.15, 0.78 mg. of liver catalase, 0.74 mg. of blood catalase/ml., cell length 4.98 mm. Horse blood catalase ●, horse liver catalase ○.

The ultraviolet absorption was earlier determined by Stern and Lavin (19). They found the absorption of 275  $m\mu$  to be twice that at 405  $m\mu$ , while Agner (20) found about the same absorption at 275  $m\mu$  and 405  $m\mu$ . The two spectra agree from 250  $m\mu$  to about 380  $m\mu$  while at 405  $m\mu$  the blood catalase has 10.6% higher absorption coefficient than the liver catalase.

### *Precipitin Reaction*

Two young adult rabbits were injected 6 times, 3 times with 2-day and 3 times with 4-day intervals. One ml. was used, for one rabbit with 9.3 mg./ml. and for the other 20 mg./ml. A week later blood was taken from the animals by bleeding from the ear.

The amount of catalase which the serum could precipitate was determined by adding a known amount of catalase to the serum and titrating the remaining catalase after centrifuging.

From 1 ml. about 0.8 mg. of both blood and horse liver catalase was precipitated. The nitrogen content of the precipitate was determined after washing with water. 0.457 mg. of nitrogen was found from the blood catalase and 0.436 mg. from the liver catalase precipitate.

Serum from a non-immunized rabbit was used as control. It did not give any reaction in the dilution 1:10.

To 1 ml. of the immune serum diluted 1:10 was added 0.01 and 0.1 mg. of human blood catalase, and of oxyhemoglobin, liver and blood catalase from horse. Only the two last gave a flocculent precipitate, the others remaining clear.

To another series of tubes horse liver and blood catalase was added in decreasing amounts from 0.48 mg. Both of the catalases gave a turbidity down to 0.000048 mg.

### DISCUSSION

As has been stated, samples of catalase from different preparations, crystallized from water, were used for the analyses. Table I shows that no appreciable variation in activity or iron and hemin content occurred from sample to sample.

The figures given for the amino acid content of liver catalase (Tables IV and V) agree as well as could be expected with those published earlier by Theorell and Åkeson (6). However, the latter authors have found 1% more arginine.

The variation in the amino acid analyses among the different samples and between horse liver and blood catalase is within the limits of error of the methods used.

Immunologically no difference was found and, in the ultraviolet, the absorption coefficient is the same.

It can thus be reasonably assumed that the protein component of the two catalases is the same, the only difference being that one hemin group/molecule in the liver catalase has been oxidized to "verdohemochromogen." When the molecule is split to protein and hemins by means of acetone-hydrochloric acid, verdohemochromogen splits off iron and gives biliverdin.

The probability of a common origin for liver and blood catalase is favored by the identity of the protein group of both substances. Perhaps liver catalase is nothing but blood catalase liberated from the red corpuscles during their destruction.

#### ACKNOWLEDGMENTS

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#### SUMMARY

The object of this investigation was to ascertain whether the protein components of horse liver and blood catalase were identical.

Methods of preparing horse liver and blood catalases and human blood catalase in a crystalline state are described. The catalases obtained from different preparations had the same properties.

The values for nitrogen distribution, histidine, arginine, lysine, tyrosine, cystine, glutamic and aspartic acids were found to agree within the limits of error for both catalases.

The ultraviolet absorption is the same from 240 to 380 m $\mu$ . At 405 m $\mu$  the hemin band of the blood catalase is a little higher than that of the liver catalase, as might be expected.

The serum of rabbits treated with horse liver catalase precipitated both horse liver and blood catalase in the same amounts. Human blood catalase and horse oxyhemoglobin as test antigen did not give any reaction.

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# Adsorption by Ion Exchange Materials of Putrefactive Chemicals

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## INTRODUCTION

Indole, skatole, histamine, guanidine and tyramine are examples of toxic chemicals produced endogenously. These compounds were considered as a group not only because of their endogenous origin but also because each is associated in the literature with diseases known to afflict the human body.

It was considered of interest to attempt to confine these chemicals to the intestinal tract introducing inert materials on which they would be adsorbed. The compounds would thus be carried through the tract without significant absorption. The main interest in this field lies in the possibility that long continued exposure of the body to even very small concentrations of such chemicals may exert a decidedly deleterious effect and may even be a factor in the disease condition referred to as aging.

## METHODS

Preliminary experiments showed that Amberlite IR-4 (anion exchange) in the alkaline range and near neutral adsorbs indole and skatole powerfully. Amberlite IR-100 (cation exchange) proved to adsorb indole and skatole only weakly from both alkaline and acid media. The problem, therefore, became the determination of adsorption data for indole and skatole by Amberlite IR-4. Two different hydrogen ion concentrations were used (pH 6.0 and 10.5) to show the effect of hydrogen ion concentration on the adsorption. These values of  $[H^+]$  represent the extremes to be found in the intestinal tract. Other values can be obtained by interpolation. It may be mentioned in passing that histamine, tyramine and putrescine are not adsorbed by Amberlite IR-4 in the pH range 6.0-10.5. There may be a degree of specificity for the indole nucleus.

The adsorption data were obtained by adding varying amounts of thoroughly washed (10 $\times$ ) Amberlite IR-4 to 100 cc. portions of 0.1% indole and 0.01% skatole



solutions brought to the indicated pH with the help of a potentiometer. After thorough shaking the filtered solutions were assayed by appropriate methods, as outlined below, and the adsorption data calculated by fitting them to a Freundlich type isotherm. The method for the determination of indole depends upon the reaction of sodium  $\beta$ -naphthoquinone-4-sulphonate with indole in alkaline solution (1). The color develops in 15 minutes and is extracted with chloroform. The cloud due to water in the  $\text{CHCl}_3$  may be removed by a small amount of anhydrous sodium sulphate. The unknown is compared with a standard solution of indole.

Reaction of skatole with trichloroacetic acid in acetic acid solution (2) gives a purple color which can be used colorimetrically. The color requires at least 24 hours to develop.

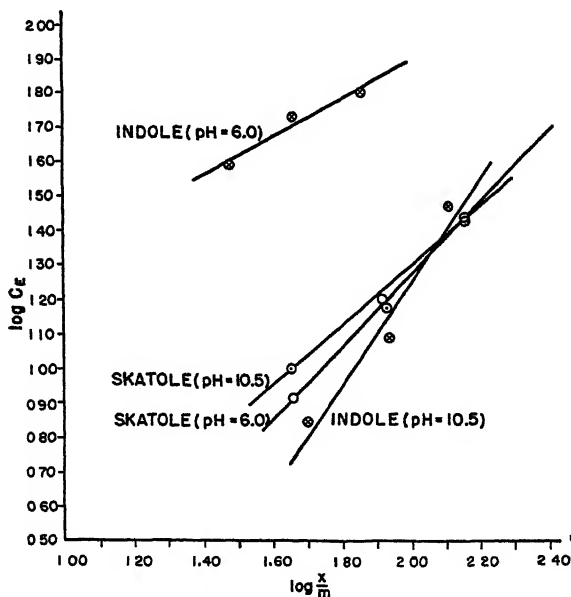


FIG. 1. Adsorption by Amberlite IR-4 of Skatole and Indole at 25°C.  
(Freundlich type isotherm) (pH = 10.5) (pH = 6.0).

### EXPERIMENTAL RESULTS

As previously shown for a number of substances adsorbed by Amberlite IR-4, the adsorption of indole and skatole fits the straight line plot of  $\log C_E$  vs.  $\log x/m$  of the Freundlich isotherm. The data are summarized in Fig. 1.

Adsorption by permutit of putrefactive chemicals was studied. *Qualitatively*, all amines tested, without exception, are strongly ad-

TABLE I  
*Adsorption by Permutit of Putrefactive Chemicals*

Compound	g./100 cc.	Adsorbed per cent
Putrescine	0.16	84
Histamine	0.25	86
Tyramine	0.29	93
Indole	0.10	> 95
Skatole	0.01	> 95

sorbed on activated permutit (activated by treatment with HCl). The adsorption is reversible at acid pH's. *Quantitatively*, Table I shows the percentage adsorptions found when 25 cc. of a solution of the given concentration were shaken with 2.0 g. permutit in the pH range 9.0-10.0.

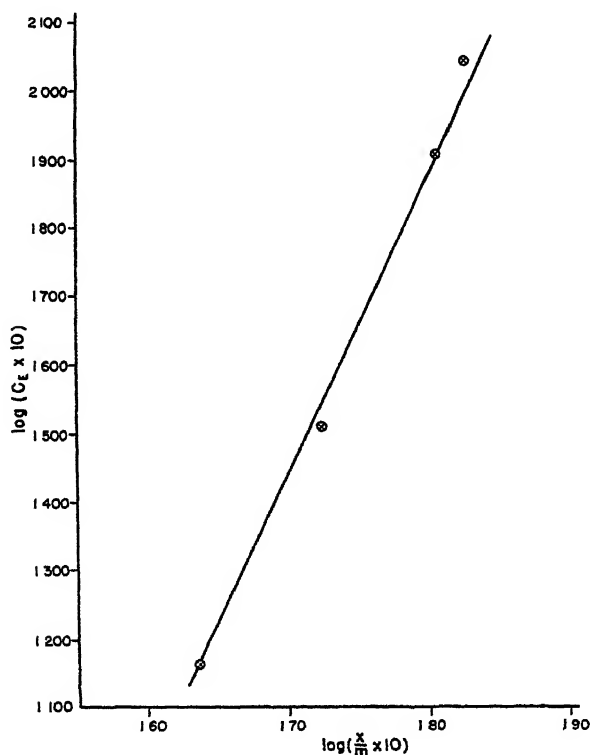


FIG. 2. Adsorption Isotherm of Guanidine by Permutit (Folin).

In addition, the adsorption isotherm was determined for guanidine. The results are given graphically by Fig. 2.

Kaolin, activated or inactivated, cannot compare with permutit as an adsorbing agent for amines. For example, with kaolin no adsorption of histamine or skatole takes place.

Three series of toxicity experiments were conducted with both indole and guanidine: The control series, a series in which the adsorbing material had been added to the basic diet at 10% and, finally, a series in which the adsorbing agent had been added to the diet and an additional quantity given with the toxic chemical. Mice (270) were used for the acute toxicity experiments. The results with the acute toxicity series are listed in the following table.

TABLE II

*Biological Results with Ion Exchange Materials and Toxic Putrefactive Chemicals*

Toxic chemical	Amount used	Adsorbing agent	Amount used	Number mice	Deaths	Controls
	<i>g./kg.</i>				<i>per cent</i>	
Indole	0.4	Amberlite IR-4	10% in diet	20	20	50%
	0.5			20	30	60%
	0.6			20	60	70%
	0.4	Amberlite IR-4	10% in diet + 5% in suspension	20	10	50%
	0.5			20	30	60%
	0.6			20	30	70%
Guanidine acetate	0.4	Permutit	10% in diet	10	10	30%
	0.5			10	30	50%
	0.6			10	40	50%
	0.4	Permutit	10% in diet + 5% in suspension	10	20	30%
	0.5			10	20	50%
	0.6			10	30	50%

Indole was administered in 5% suspension and guanidine acetate in 5% solution.

The reduction in the acute toxicity of indole and guanidine is interpreted as indicating adsorption of the toxic chemicals on the inert and non-adsorbable agent which confines the toxic chemical to the intestinal tract.

## DISCUSSION

In the adsorption equation proposed by Freundlich (3) (where  $x$  is

$$\log x/m = \log a + k \log C_E,$$

the amount of indole or skatole adsorbed by  $m$  g. of Amberlite from a solution whose equilibrium concentration is  $C_E$ ), the constant  $k$  is given by the slope of the line represented by the above equation. As indole and skatole are so much alike it is not surprising that the slopes of the lines are very close in numerical value as is apparent from Fig. 1. There is a concentration of Amberlite beyond which very little further adsorption takes place. Thus the administration of 1 g., or even less, of Amberlite will have very nearly the same physiological effect as 2 g. Although the adsorption of skatole is practically identical at pH 6.0 and pH 10.5, the adsorption of indole is considerably less at pH 6.0.

The failure of Amberlite to adsorb histamine, tyramine and putrescine suggested the necessity of seeking an agent capable of adsorbing these materials. Permutit proved highly successful. It was effective in adsorbing indole and skatole in addition to the 3 chemicals which were not adsorbed by Amberlite, namely: histamine, tyramine and putrescine. It is probable, therefore, that efforts to restrict chemicals of a toxic nature to the intestinal tract should involve the use of several agents with selective action so that they will not remove beneficial agents such as vitamins. Combinations of Amberlite and permutit would seem to offer a possibility.

Amberlite IR-4 showed (4) no adsorption of thiamin or riboflavin at the pH of the normal human stomach or intestinal tract. The resin strongly adsorbed ascorbic acid at the pH of the stomach, but desorption was complete at the pH of the normal intestinal tract. Furthermore, rat feeding experiments showed no defective nutrition when 5% of resin was fed in the diet. Preliminary results with feeding tests (stock diet plus 5% permutit) have failed in 6 weeks to disclose any removal of nutritionally important chemicals. Melnick *et al.* (5) have reported that fuller's earth, but not kaolin, markedly reduced the availability of thiamin.

The problem is one of differential adsorption in a slightly alkaline medium. The pH of the small and of the large intestine would vary from 7 to nearly 10. The adsorbing agents selected would need to remove the toxic chemicals at this pH and yet permit absorption of nutritionally important agents. Amberlite IR-4 meets this require-

ment with all substances so far tested, but fails to adsorb amines. Permutit would also seem to answer the requirements.

Medically, the satisfactory solution to the problem of restricting toxic chemicals to the gut and permitting the absorption of nutrients is of tremendous importance. Diarrhea, and perhaps constipation, may in some cases be due to toxic chemicals. Adsorbing agents (kaolin) have already found a place in medicine in the treatment of infantile diarrheas.

The biological results disclose the effectiveness of permutit and Amberlite in causing a relative restriction of the adsorption *in vivo* of the toxic agents studied. The death rate from indole and guanidine was materially reduced. Furthermore, the blood levels of indole showed significant differences, a finding which supports the suggestion that inert adsorbing agents can be used effectively in keeping these toxic chemicals confined to the intestinal tract.

The ineffectiveness of kaolin, and the demonstration that multiple adsorbing agents must be employed for effective action in the tract, strongly suggest that presently employed preparations for adsorption are not really effective. The amount of indole in the feces is normally 50–60 mg., while some 12–20 mg. of indole (as indican) is excreted in the urine. Reduction of the amount of indole and similar chemicals absorbed from the intestinal tract may be a factor in the disease condition referred to as aging. We know virtually nothing about the effect of long continued exposure to low concentrations of toxic chemicals, but it seems reasonable that elimination of this factor would favorably influence the course of health and longevity.

#### SUMMARY

Physicochemical and biological studies have been reported showing the effectiveness of ion exchange materials in confining to the intestinal tract certain putrefactive chemicals. Amberlite IR-4 was shown to be effective against indole and skatole, while permutit was effective against putrescine, cadaverine, tyramine, histamine and guanidine. The possibility of the application of these results to medical practice has been considered.

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# The Absorption of Niacin by Yeast

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## INTRODUCTION

Considerable attention has been given recently to methods of increasing the vitamin content of yeast intended for food, baking and pharmaceutical purposes. Niacin values for yeasts vary appreciably, even within specific types, indicating that conditions of cultivation and the composition of the medium influence the potency of the cells (1, 2, 3). Grain alcohol fermentation and other process waste liquors are rich in niacin and the recovery of this vitamin in the form of fortified yeast might be practical. In the present study, the role of absorption in determining the niacin content of yeast has been investigated. The ability of various yeasts to absorb niacin and some factors affecting the niacin content of bakers' yeast have been determined.

## METHODS

Cultures employed, except for the strains of bakers' yeast, were from the Culture Collection of the Northern Regional Research Laboratory. The two cultures of bakers' yeast were isolated and purified from a 1-pound cake of commercial high-thiamine yeast. A beet molasses-malt extract medium was used (4) in most of these experiments. This medium contained approximately 4.5% total sugar and 0.16% nitrogen. Its niacin content varied from 4.5 to 4.8 mg./liter when assayed without hydrolysis. For certain experiments the synthetic medium of Williams *et al.* (5) was modified by substituting 40 g. of glucose/liter for the sucrose, reducing the ammonium sulfate to 0.5 g./liter, and adding 3.0 g. of urea/liter. Media were dispensed in 100 and 300 ml. quantities in 500 ml. flasks for aerobic and anaerobic cultivation, respectively. After sterilization and cooling, flasks were seeded with sufficient inoculum to provide 0.15 g. of moist yeast/100 ml. of medium. The inoculum was produced on molasses-malt

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extract medium described above and was incubated for 24 hours. Cells inoculated into synthetic medium were previously washed by centrifugation. Aerobic cultures were shaken continuously and anaerobic cultures were incubated without agitation.

After 48 hours at 30°C., the cell crops were determined by weighing the washed cells. The cells were then resuspended and aliquots of the suspension were taken for determination of niacin content and dry substance. Niacin was determined by the method of Snell and Wright (6), nicotinic acid being used as the standard. Cells were digested for 30 minutes at 121°C. in the presence of 1 *N* sodium hydroxide; media, unless stated otherwise, were assayed without prior treatment. Dry substance was measured by drying overnight at 105°C. Yeast assays are reported on the dry basis.

### EXPERIMENTAL

*Absorption of Niacin by Various Yeasts.* Ten yeast cultures were investigated for their ability to absorb niacin during cultivation on molasses-malt extract medium. The cultures selected represented bakers', brewers', distillers' grain, distillers' molasses and food yeast types. Also included were *Candida guilliermondia* which synthesizes substantial amounts of riboflavin (7) and *Torulopsis lactosa* which may be distinguished from the other types mentioned above by its inability to synthesize niacin (8).

The distribution of niacin between cells and media after 48 hours' incubation is shown in Table I. From these data it may be seen that yeasts are capable of absorbing a considerable proportion of niacin from the unsupplemented medium. The niacin contents of the cells varied from 0.31 to 0.515 mg./g. of dry substance. *C. guilliermondia*, distillers' molasses yeast, and *T. lactosa* were the highest with 0.515, 0.466 and 0.447 mg./g., respectively. The distillers' molasses type differed from the other yeasts in that it absorbed niacin poorly, but nevertheless attained a high potency, indicating unusual synthetic ability. Despite synthesis, which undoubtedly occurred, there was, in most cases, a small net loss of niacin. The niacin content of only two cultures (Y-324 and Y-1347), in cells and fermented medium, was as much as, or more than, in the unfermented medium. The lowest recovery of niacin was found with the two top yeasts (Y-663 and Y-647) for which only 73 and 75% of the vitamin was accounted. Other than the breakdown of niacin by yeasts as noted above, it has been shown (9) that *Proteus vulgaris* destroys nicotinic acid during its active metabolism while certain strains of *Pseudomonas* and *Serratia* have been found capable of utilizing nicotinic acid when it is the sole source of carbon (10).

In the basal medium supplemented with 10 mg. of niacin/l., the average potency of the cells was 0.688 mg./g. compared with 0.366

TABLE I  
*The Absorption of Niacin by Various Types of Yeast*

Yeast type	NRRL culture	Basal medium (4.65 mg. niacin/l.)				Basal medium supplemented with niacin (14.65 mg. niacin/l.)			
		Yield of dry yeast	Niacin in cells		Niacin in fermented medium	Yield of dry yeast	Niacin in cells		Niacin in fermented medium
			mg./lb.	Per cent of total in unfermented medium			mg./lb.	Per cent of total in unfermented medium	
		g./l.				g./l.			
Distillers' grain	Y-567	8.3	.310	56	31	8.3	1.280	72	22
Distillers' molasses	Y-1347	7.7	.466	78	65	7.3	.505	25	75
Brewers' top yeast	Y-663	5.3	.312	36	39	5.6	.326	12	72
Brewers' top yeast	Y-647	6.6	.310	44	29	6.2	.447	19	71
Bakers' "A"		8.4	.310	56	32	8.1	1.050	58	41
Bakers' "B"		8.2	.315	56	31	8.4	.947	55	39
<i>Torulopsis utilis</i>	Y-900	8.0	.304	52	33	8.2	.516	29	50
<i>Torulopsis utilis</i>	Y-1084	7.7	.334	55	28	7.9	.780	42	40
<i>Torulopsis lactosa</i>	Y-168	6.7	.447	63	33	7.0	.480	23	69
<i>Candida guilliermondia</i>	Y-324	6.4	.515	70	40	6.4	.550	24	62



mg./g. for the same cultures grown on the unsupplemented basal medium. Cell levels varied from 0.326 mg./g. for top yeast Y-663 to 1.28 mg./g. for grain distillers' yeast, Y-567. With the latter culture, the cells contained vitamin equivalent to 72% of that present in the unfermented medium. The bakers' yeasts followed in efficiency of absorption.

*Absorption of Niacin under Aerobic and Anaerobic Conditions.* Bakers' yeast was inoculated into flasks of molasses-malt extract medium with and without added niacin. The flasks were incubated under aerobic and anaerobic conditions, and niacin was determined in cells and media after 24, 48 and 72 hours.

TABLE II

*Absorption of Niacin by Bakers' Yeast (Strain B) Cultivated under Aerobic and Anaerobic Conditions*

Period of incubation	Basal medium (4.85 mg. niacin/l.)				Basal medium supplemented with niacin (15.15 mg. niacin/l.)			
	Yield of dry yeast	Niacin in cells		Niacin in fermented medium	Yield of dry yeast	Niacin in cells		Niacin in fermented medium
Hours	g./l.	mg./g.	Per cent of total in unfermented medium	Per cent of total in unfermented medium	g./l.	mg./g.	Per cent of total in unfermented medium	Per cent of total in unfermented medium
<i>Aerobic</i>								
24	6.2	.376	48	32	6.2	1.017	41	44
48	7.6	.320	50	31	7.6	.850	43	44
72 (a) <sup>1</sup>	8.5	.290	51	48	8.8	.755	44	51
(b)					8.6	.600	34	54
<i>Anaerobic</i>								
24	2.3	.922	44	34	2.4	1.973	31	54
48	2.6	.788	42	38	2.8	1.622	30	53
72 (a) <sup>1</sup>	2.7	.722	40	45	3.0	1.150	23	62
(b)					2.6	.841	14	73

<sup>1</sup> In series (a) niacin was added at the time of inoculation; in series (b) niacin was added after 48 hours of incubation.

It may be seen from Table II that cells grown either aerobically or anaerobically showed their highest niacin potency at 24 hours. With longer incubation, the niacin level in the cells decreased. Niacin in the medium was lowest during the first 48 hours, indicating that niacin is absorbed during the period of most active cell multiplication and is later released.

The niacin content of anaerobically-produced cells was markedly higher than that of aerobically-produced cells in both fortified and un-

fortified media. However, because of the larger cell crop produced aerobically, the total amount of niacin absorbed was greater under aerobic conditions. Anaerobic cells in the fortified medium contained 1.973 mg. of niacin/g. after 24 hours' incubation. Niacin added to the medium after the cultures had been incubated for 48 hours was absorbed much less efficiently than when added before inoculation. Behavior toward niacin is different, therefore, than toward thiamine where absorption is equal, or slightly better, with non-proliferating cells (4).

*Absorption of Niacin from Synthetic Medium.* To ascertain the importance of synthesis and absorption in determining the niacin content of yeast, bakers' yeast was cultivated in synthetic medium and synthetic medium fortified with niacin. Cultures were incubated aerobically and anaerobically for 48 hours. Results (Table III) show that

TABLE III

*The Niacin Content of Bakers' Yeast (Strain B) Cultivated in Synthetic Medium with and without Added Niacin*

Niacin in unfermented medium	Yield of dry yeast	Niacin in unfermented medium	Niacin in cells	
			mg./g.	Per cent synthesized
<i>Aerobic</i>				
0	4.6	1.38	.585	100
10.85	4.6	7.55	1.24	53
31.20	4.5	27.6	1.35	43
<i>Anaerobic</i>				
0	1.7	0.11	.190	100
10.85	1.7	6.94	1.29	15
31.20	1.8	26.6	1.65	11

cells grown aerobically without added vitamin are approximately three times as rich in niacin as those grown anaerobically. Also, they excreted into the medium, per g. of cells produced, more than four times as much niacin as anaerobically-grown cells. In the presence of added niacin, anaerobic cells were somewhat richer in niacin due to absorption from the medium. If it is assumed that added niacin did not influence synthesis, then 85-90% of the potency of anaerobic cells was due to

absorption, while approximately 50% of that in aerobic cells was absorbed.

Yeast cultivated aerobically on synthetic medium developed an appreciably higher niacin content than cells produced on molasses-malt extract medium. This is not generally true with other B vitamins, and some experiments were performed to confirm this observation. It was found that cell niacin was increased by increasing the asparagine content of the synthetic medium and decreased markedly by supplementing synthetic medium with Difco yeast extract or molasses-malt extract medium.

*Effect of Aeration on Niacin Retention.* The above experiments, which demonstrated an absorption of niacin, were carried out with shaken cultures and with much longer periods of incubation than are commonly used in commercial yeast production.

The following experiment was performed in larger vessels aerated with a stream of air and from which samples could be frequently withdrawn. Niacin was added to raise the level of molasses-malt extract medium to 25.9 mg./liter. To one two-liter portion was added commercial bakers' yeast (pound goods) to supply 27.0 g. of dry yeast and to the other portion was added the same yeast to supply 4.0 g. of dry cells. The first portion was incubated anaerobically and the second was aerated by means of a perforated aluminum tube sparger at the rate of 3 liters of humidified air per minute. The temperature was maintained at 29°-30°C. and samples were removed after 2, 4, 8, 12 and 24 hours for determinations of residual sugar and niacin in cells and medium.

During the 24-hour period the cell weight increased from 4 to 22 g. under aerobic conditions and from 27 to 29.2 g. under anaerobic conditions. The utilization of sugar and the distribution of niacin between cells and medium are shown in Fig. 1. Both aerated and unaerated cells began to increase in niacin potency almost immediately. From an initial content of 0.61 mg./g., aerated and unaerated cells attained values of 1.15 mg. and 1.00 mg./g., respectively, within 2 hours. Aerated cells thereafter increased slowly to their final content of 1.21 mg./g. Unaerated cells, on the other hand, reached their highest content (1.24 mg./g.) at 4 hours, after which the niacin level decreased sharply with some vitamin reappearing in the medium. The high rate of niacin absorption during sugar utilization and its release by the cells after the reducing sugar had reached a low and nearly constant level is of considerable interest. Since only a part of the niacin released from the cells can be found in the medium, even after digestion with alkali,

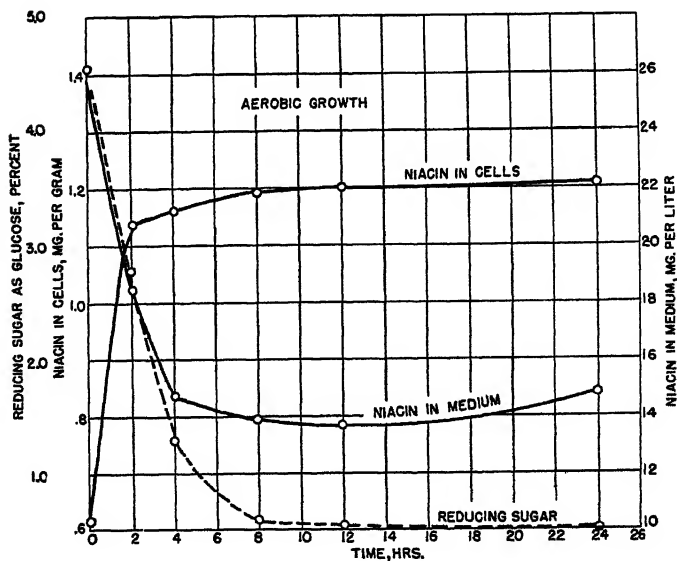
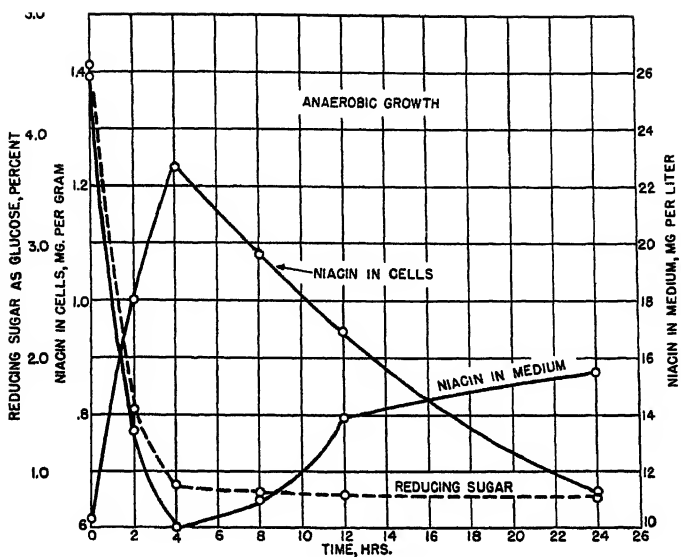


FIG. 1. The Effect of Aeration upon the Absorption and Retention of Niacin by Bakers' Yeast (Strain B)

destruction of the vitamin must be assumed. Aerobically cultivated cells released niacin only during the later stages of the incubation period and then in much smaller quantities.

*Absorption of Niacin Occurring in Natural Products.* The ability of yeast to absorb niacin from wheat stillage and rice bran extract (Vitab) was investigated. Both of these materials are good sources of niacin and each contains approximately one-half the niacin in the bound form; *i.e.*, available to the assay organism only after digestion with 1 *N* alkali. Vitab was added with and without alkali treatment at the rate of 5 ml./liter of molasses-malt extract medium. Media were also prepared which contained 50 g. of beet molasses/liter of clarified stillage. One medium contained alkali-treated stillage. Bakers' yeast was cultivated aerobically in all of these media for 48 hours.

The results (Table IV) show that alkali treatment greatly improved the absorption of niacin, giving cells of higher niacin content. However, in all of these trials, as well as in earlier experiments, niacin was never completely removed from the fermented medium.

## DISCUSSION

From the experiments reported herein, it is apparent that niacin is taken up from the medium by both growing and fermenting yeasts, although with somewhat less avidity than is thiamine. Yeast strains vary considerably in their capacity to take up niacin; the top brewers' yeasts (Y-647 and Y-663) and the molasses distillers' yeast (Y-1347) showed the lowest efficiency, while grain distillers' yeast (Y-567) was exceptionally good in fortified media. The bakers' yeasts also removed more than half of the niacin from fortified media.

Niacin is strongly absorbed during the active fermentation of carbohydrate under anaerobic conditions. After the fermentable carbohydrate has been metabolized, niacin is released and partly destroyed. This observation is in contrast to the finding of Norris (11) who was unable to demonstrate either absorption or excretion of niacin after incubation of brewers' top yeast for 10 to 14 days. Morel (9) noted that nicotinic acid amide was destroyed during the active metabolism of *Proteus vulgaris*. The destruction of niacin observed in our work with bakers' yeast was probably accentuated by the large inoculum and by the low concentration of fermentable sugar.

With brewers' yeast and other anaerobically propagated yeasts, the

TABLE IV  
*The Absorption of Niacin from Rice Bran Extract (Vitab) and Wheat Stillage by Baker's Yeast (Strain B)*

Medium	Niacin supplement	Treatment of supplement	Free niacin in medium		Niacin in cells	
			Unfermented	Fermented	mg lb.	Per cent of niacin in unfermented medium
			mg./l.	Per cent of total in unfermented medium		
Molasses-malt extract	None	None	5.15	41	.306	49
Molasses-malt extract	5 ml. Vitab/l.	None	11.22	57	.515	37
Molasses-malt extract	5 ml. Vitab/l.	Hydrolyzed with 1 N NaOH	15.00	21	1.145	61
Molasses	Wheat stillage	None	7.58	46	.387	35
Molasses	Wheat stillage	Hydrolyzed with 1 N NaOH	10.35	27	.978	62

higher niacin levels are probably due largely to absorption. This is analogous to the behavior of yeasts toward thiamine. Aerobically propagated yeast often attains a high niacin potency which appears due, to a considerable extent, to synthesis. This was demonstrated in the synthetic medium where, under aerobic conditions, the niacin content of bakers' yeast was significantly higher than that obtained by culturing the same yeast in molasses-malt extract media. The molasses-malt extract medium and yeast extract were found to depress niacin synthesis. The same factors present in these materials may be responsible for the variations in niacin content of commercial yeasts.

Niacin in natural products, such as the rice bran extract and wheat stillage which were investigated, occurs in at least two forms, only one of which is readily taken up by yeast. Recovery of niacin from such products would require hydrolysis prior to fermentation.

#### ACKNOWLEDGMENT

The author wishes to express his appreciation to Shirley E. Pfeiffer for technical assistance during the course of this investigation.

#### SUMMARY

The ability of various yeasts to take up niacin from unfortified and fortified molasses-malt extract media has been investigated. In unfortified media, *C. guilliermondia*, *T. lactosa*, and a strain of molasses distillers' yeast showed higher than average niacin contents. In fortified media, a strain of distillers' grain yeast and commercial bakers' yeast attained the highest cell potencies. The grain yeast removed nearly three-fourths of the niacin present in the original medium.

Of the niacin contained in bakers' yeast cultivated anaerobically on synthetic medium, 85% or more was absorbed from the medium, while only 50% of the vitamin from aerobically-propagated cells came from the medium.

When media are supplemented with naturally-occurring products containing niacin, absorption is markedly enhanced by prior digestion of the supplement with alkali.

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# Crystalline Human Myoglobin from Heart-Muscle and Urine

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## INTRODUCTION

Since one of us (16, 17, 18, 19, 20) first prepared crystalline horse myoglobin and investigated some of its properties, this substance has been extensively studied by a number of authors. Numerous data, most of which may be found in Millikan's review on the subject (13), have been obtained on the physiological and biochemical properties of the pigment, emphasizing the differences between myoglobin and hemoglobin. It has become quite clear that, although both pigments contain the same proportion of an indetical hem, this is combined in myoglobin with a globin of much lower molecular weight and different composition than that in hemoglobin.

Most of the data accumulated until now have been obtained on horse myoglobin, but the myoglobins of other animal species have also been studied and evidence of specific differences, such as occur between the hemoglobins of different origin, has been secured. Most of these comparative studies, however, have been made on crude muscle extracts and it is only recently that complete purification of various myoglobins has been attempted by Roche, Derrien and Vieil (15). Their investigations have revealed the fact that horse myoglobin and that of closely related animals, such as the donkey and the mule, are by far the easiest to obtain in the crystalline state. They succeeded with more difficulty in crystallizing oxymyoglobin, but could only get amorphous preparations from dog, pig and sheep muscle. Except for some observations made on unpurified solutions by Haurowitz (11), human myoglobin seems to have been but little studied in earlier years. It has lately been detected by Bywaters (2, 4, 5) in the urine of air raid casualties suffering from crushing injuries and, together with acidosis, has been held responsible by this author for the consecutive renal failure observed on these patients. To study this phenomenon more closely on rabbits, Bywaters and Stead (6) have prepared partly purified solutions of myoglobin extracted from human muscle but did not attempt to crystallize the pigment.

The present paper deals with the preparation of crystalline human myoglobin extracted both from heart muscle and urine. The urine samples proved very helpful, as they were entirely hemoglobin-free. They were furnished by two cases of mild paralytic myoglobinuria, occurring in two brothers who, for a number of years, have excreted myoglobin as a result of any severe muscular strain. It does not enter the scope of this paper to discuss the clinical aspect of this disease,<sup>1</sup> which is well known and has been fully studied in the horse by Carlström (7), and has occasionally been diagnosed in man. However, it may be interesting to mention, since it has been newly claimed by Grzycki and Gucfa (10) that horse myoglobinuria is associated with carbohydrate metabolism deficiencies which can be restored by insulin (see also Carlström (7)), that, when put on a carbohydrate-free diet, one of the brothers developed an extremely severe attack of myoglobinuria. The urine excreted during this attack contained large amounts of myoglobin and served for the extraction described in this paper. Although the largest part of the pigment was destroyed as a result of bacterial fermentation, it was possible to isolate a small fraction of it in crystalline form and to compare it with the crystalline myoglobin prepared from heart-muscle extract.

### PRELIMINARY INVESTIGATIONS

The isolation of myoglobin is complicated by the presence of large quantities of hemoglobin in the extracts. Several methods have been advocated for separation of the two pigments. The simple method of perfusing the organ with isotonic NaCl and washing it fairly free of blood, which has been used by Theorell and other authors in the preparation of myoglobin from horse heart, was of course no longer applicable in the present case. By quickly washing the muscle pulp after mincing, Haurowitz (11) claims to have eliminated most of the hemoglobin without loss of myoglobin from a preparation of human muscle, but the method seems untrustworthy and difficult to apply in a large scale experiment. According to Bywaters and coworkers (4), the smaller volume of the myoglobin molecule makes it possible to separate it from hemoglobin by ultra-filtration, but not without losses. Here again this elegant method seemed hardly adapted to the treatment of several liters of solution. A general method for separating myoglobin from hemoglobin in extracts of unwashed muscle has been described by Roche, Derrien and Vieil (15). It is based on the more practical but tedious procedure of repeatedly fractionating the extract with ammonium sulfate and controlling the purity of the active fraction by making a solubility curve according to Cohn (8). It is rendered possible by the fact, first demonstrated by

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<sup>1</sup> The clinical aspects of these cases will be published by Dr. R. Hed, Garnisons-sjukhuset, Sollefteå, Sweden.

Morgan (14) on the horse pigment, and further confirmed for the horse as well as for other animal species by Roche and coworkers (15), that, even though important differences occur from species to species, hemoglobin is always salted out at lower concentrations than the myoglobin of the same animal. A similar method was adopted in the present case after its practicability had been examined.

A preliminary investigation carried out on fresh laked blood showed that the salting out of human oxyhemoglobin occurs between ammonium sulfate concentrations of 70–75% saturation. Addition of ammonium sulfate, up to 75% saturation, to an extract of human heart-muscle, prepared and partly purified according to Theorell (16) resulted in the precipitation of a large part of the hemoglobin present without apparent loss of myoglobin; but the presence of small amounts of hemoglobin could still be detected spectrophotometrically in the filtrate. By further increasing the concentration of ammonium sulfate, it was possible to remove the residual hemoglobin, but only together with a certain amount of myoglobin. Apparently, the salting-out constants of the two pigments are not sufficiently different to allow a quantitative separation to be made, but the main fact was that hemoglobin could be quantitatively removed if only at the cost of a loss in myoglobin. This was further checked by submitting a filtrate believed to be hemoglobin-free to 6 hours ultracentrifuging at 35,000 r.p.m. and analyzing spectrophotometrically, after reduction and saturation with CO, the upper and lower layers of the centrifuged solution. The fact that no difference was observed in the relative light-absorption capacities of the two layers showed the absence of hemoglobin in the solution. By adding varying quantities of hemoglobin to hemoglobin-free solutions of myoglobin and fractionating the mixture with ammonium sulfate it was further shown that even dilute solutions of myoglobin could be completely freed in this way from large quantities of hemoglobin.

## METHOD OF PREPARATION

### *A. Sample 1 from Heart Muscle Extract*

The first steps were carried out according to the method described by Theorell (16) for the preparation of horse myoglobin. Twenty human hearts, collected from post-mortem examinations on twenty non-infectious cases, were roughly dissected free of fat and connective tissue and mechanically minced, when 2,500 ml. of distilled water were added to the 2,500 g. of pulp obtained. The mixture was then vigorously stirred and allowed to stand overnight in the cold. The cell-residues were centrifuged off and particles of congealed fat which had accumulated on the surface of the liquid removed, whereupon 3,360 ml. of a slightly turbid, red-colored extract, pH 6.15, were obtained. The pH was adjusted to 7 with *N* NaOH and 840 ml. of *M*/2 basic lead acetate slowly added to the extract at room temperature, with continuous stirring, creating a voluminous greyish-white precipitate, containing, among other impurities, all the muscular globulins. It was then necessary to readjust the slightly lowered pH with *N* NaOH, causing a certain amount of additional precipitate to be formed. The precipitate was centrifuged off and the excess lead then removed by careful addition of small amounts of solid sodium phosphate. The reaction of the solution is apt to acidify strongly under this treatment through liberation of free acetic acid, and it was necessary to check the pH after each new addition of phosphate. By using pure tertiary phosphate,

it was possible to keep the pH continuously in the neighborhood of 7, but a careful watch had to be kept on the completion of the reaction as an excess of tertiary phosphate will result in a strong increase of the pH. Considering myoglobin is much more resistant toward bases than toward acids, this procedure was judged safer and the increase of pH to a value of 8 which occurred after complete precipitation of the lead was quickly neutralized by adding a few crystals of primary phosphate. The lead phosphate precipitate was centrifuged off and 4,100 ml. of a clear cherry-red solution were obtained. This solution was then evaporated *in vacuo* down to a volume of 500 ml. and subsequently saturated with carbon monoxide after reduction with  $\text{Na}_2\text{S}_2\text{O}_4$ .

Before starting fractionation with ammonium sulfate, it was, of course, necessary to estimate first the amount of salt already present in the concentrated solution. A rough calculation showed that the ionic strength should be about 2.5 and, in view of this fact, the first fraction was obtained by adding solid ammonium sulfate to the solution up to 50% saturation instead of 75%, which would have been necessary if no salt had been previously present in appreciable amounts. In fact, most of the hemoglobin precipitated at this point and was filtered off. The filtrate was then successively brought to 55, 60 and 65% saturation by adding further amounts of ammonium sulfate, the precipitate being filtered off after each new addition of salt and samples of each filtrate being kept aside as well. The various precipitates were redissolved in *M*/15 phosphate buffer, pH 7, and each filtrate as well as each redissolved precipitate was once again saturated with CO after reduction with  $\text{Na}_2\text{S}_2\text{O}_4$  and examined spectroscopically as to the position of the light-absorption maximum between 570 and 580  $\text{m}\mu$ . For the last two samples, the results were confirmed by a more accurate determination, using the spectrophotometric technique further described in this paper. As a further check, similar determinations were made on two samples of myoglobin-containing urine. No difference was observed in the positions of the  $\alpha$ -band of the fraction precipitated between 60 and 65% saturation, the filtrate from this fraction and the two urine-samples, showing that all the hemoglobin had been precipitated at a saturation of 60%. For practical purposes, it must be pointed out that the values of ammonium sulfate saturation given above have a purely relative value and are dependent on the concentration of salt already present in the solution.

To eliminate the more soluble impurities, the concentration of ammonium sulfate in the hemoglobin-free filtrate was brought to 100% saturation, causing the precipitation of the larger part of the myoglobin. This was filtered off, washed on the filter with saturated ammon-

ium sulfate and finally redissolved in 100 ml. *M*/15 phosphate buffer, pH 7. Ammonium sulfate was removed by dialysis in cellophane tubes, first against running distilled water containing 0.1%  $\text{NH}_3$ , and then against *M*/20 phosphate buffer, pH 7. The volume was brought back to 100 ml. by evaporation *in vacuo* and the solution again dialyzed in the cold against a carefully controlled *M*/20 phosphate buffer, pH 6.9. The myoglobin solution was then submitted to a 3 days cataphoresis according to Tiselius. A yellowish substance was observed wandering rapidly towards the anode. No attempt was made to identify this substance, but it may be of some importance, as a similar impurity was found moving off during cataphoresis of the urine myoglobin as well. It is also possible that this substance may be responsible for the fact that some myoglobins are difficult to crystallize, as it was not possible to obtain crystals from two samples which had not been purified by cataphoresis. After cataphoresis, crystallization in fine needles was easily obtained by dialyzing the solution, which by that time contained the myoglobin in the form of metmyoglobin, against saturated ammonium sulfate. The crystals were centrifuged and washed several times with saturated ammonium sulfate and subsequently redissolved by adding an equal volume of water to the suspension. A little material remained undissolved and was centrifuged off. The solution was dialyzed against increasing concentrations of ammonium sulfate. Up to 80% saturation, no crystallization occurred, but a fairly large amount of precipitate fell out which was found to consist mainly of denatured myoglobin. By increasing the concentration of ammonium sulfate to 85% saturation, most of the myoglobin remaining in solution crystallized (Fig. 1.). The crystals were separated from the mother liquor and washed first with 85%, finally with 100% saturated ammonium sulfate. Data concerning some of the determinations carried out will be summarized later in this paper.

### *B. Sample 2 from Urine*

The myoglobin-containing urine was treated in essentially the same manner as the heart-extract: Purification with basic lead acetate and removal of the lead with phosphate; fractionation with ammonium sulfate, practically the whole material precipitating between 75 and 100% saturation; dialysis; concentration by evaporation *in vacuo*; cataphoresis; dialysis against saturated ammonium sulfate. Unfortunately, a large part of the material was lost through bacterial contamination and only a small quantity of crystals, amounting to approximately 25 mg. was finally obtained (Fig. 2.), so that no recrystallization was attempted. As has already been mentioned,

upon cataphoresis, a yellowish substance, similar to that observed with the muscle extract, was seen swiftly moving toward the anode.

### OBSERVATIONS

The data recorded in this part will only be mentioned inasmuch as they support the contention that the two substances isolated from human heart and urine are identical. In view of the scarcity of the material obtained thus far, a detailed study of the properties of human myoglobin has not yet been attempted.

#### *A. Crystal Shape*

Figs. 1 and 2 show photomicrographs, taken in polarized light between crossed nicols, of the crystals of metmyoglobin extracted respectively from heart and urine. In both samples the crystals were



FIG. 1. Crystals Prepared from Heart-Muscle, between Crossed Nicols.  $\times 65$ .

needle-shaped and clearly double-refracting. But whereas the urine crystals appeared very clearly both between parallel and crossed nicols and showed a strong tendency to associate in typical fan-shaped clusters, similar to those formed by horse myoglobin, the heart crystals absorbed and refracted light very poorly and did not seem to be grouped in the same manner. It is very doubtful, however, whether these differences have any significance, and they can be probably ascribed to a certain degree of instability in the heart-preparation, due to the protracted time taken for its purification. It must indeed be

noted that typical clusters were observed after the first crystallization and that the operations performed subsequently showed an increasing tendency of the product to spontaneous denaturation. Quite a large amount was denatured during the second crystallization and an attempt to recrystallize the product a third time, after first having dialyzed it free of ammonium sulfate and subjected it to a new cataphoresis, resulted in complete denaturation upon dialysis against 85% ammonium sulfate.



Fig. 2. Crystals Prepared from Urine, between Crossed Nicols.  $\times 65$ .

### *B. Iron Content*

Simultaneous iron and dry-weight determinations were carried out on aliquot parts of solutions and on small quantities of washed, heat-coagulated material from both samples. Iron was estimated photometrically as the sulfosalicylic acid compound in ammonia solution, according to the method of Agner (1).

A mean iron content of 0.34% was found in both samples, corresponding to that of horse myoglobin and human hemoglobin.

### *C. Hemochromogen Reactions*

Treated with pyridine and sodium hydrosulfite in  $N/10$  NaOH, both samples showed the typical hemochromogen spectrum. It may be in-



teresting to mention, in view of the various interpretations put forward for this phenomenon, that a typical 4-band spectrum, such as has been described by Bechtold and Pfeilsticker (3), was obtained by the addition of a few drops of pyridine to a neutral solution of recrystallized myoglobin, in the presence of  $\text{Na}_2\text{S}_2\text{O}_4$ . By increasing the amount of pyridine, the spectrum was resolved in the normal hemochromogen spectrum. The same observation has been made on crystalline horsemyoglobin by Kiese and Kaeske (12). Gonella and Vannotti (9) have recently shown that the Bechtold spectrum is produced by a mixture of 2 different hemochromogens and conclude from this fact that only part of the hemin in the myoglobin molecule is chemically bound to the globin component, the other part being only adsorbed to the molecule. It may be wondered whether such an interpretation might still hold true for a crystalline substance.

#### *D. Light-Absorption Curve of the CO-Compound*

A series of spectrophotometric measurements were made between 630 and 500  $\text{m}\mu$  on solutions of both the heart and urine myoglobins after conversion into their carboxy compounds. The apparatus described by Warburg and Negelein (21) was used. Moreover, to prevent reoxidation to metmyoglobin and displacement of carbon monoxide by oxygen, a special device was used by which the solution was reduced with  $\text{Na}_2\text{S}_2\text{O}_4$ , saturated with CO, and finally led into an air-tight cuvette, in a pure CO atmosphere. (Fig. 3.)

The solution of metmyoglobin was placed in the modified Thunberg tube A, containing  $\text{Na}_2\text{S}_2\text{O}_4$  in the side-vessel. CO was then allowed to bubble through the solution for 15 minutes, removing the oxygen present in the solution and in the apparatus. The tube A was then rotated to bring the solution in contact with the hydrosulfite, causing instantaneous reduction of the metmyoglobin. The reduced myoglobin, in turn, rapidly combined with carbon monoxide, which was allowed to bubble through the solution for 5 minutes more to ensure complete saturation. The tube A was then rotated in the opposite direction and the solution of CO-myoglobin was forced by the slight extra pressure of the carbon monoxide through the side tube of the stopper into the air-tight cuvette B. This consisted of an ordinary 1 cm. cuvette, to which a cap of plexiglas fitted with two thin glass tubes had been adapted and entirely sealed with paraffin. The inlet

tube plunged directly to the bottom of the cuvette and was made to curve along the wall so as not to interfere with the passage of light, whereas the outlet tube was made as short as possible and curved upward into a blind hole bored in the inner surface of the cap. Both tubes were connected to the apparatus by short rubber tubes. As soon as the cuvette was completely filled and some of the solution had started flowing through the outlet tube, clamps were placed on the rubber tubes and the cuvette was disconnected from the apparatus. Under

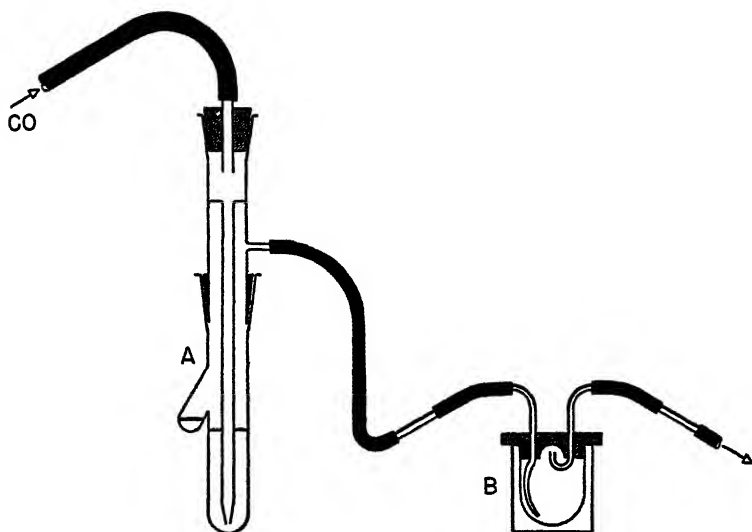


FIG. 3. Apparatus Used for the Preparation and Spectrophotometric Analysis of Carboxymyoglobin.

those conditions the solution could be kept perfectly stable for several hours, after which time no reduction of the absorption at the peak of the  $\alpha$ -band and no significant increase of the absorption at  $630\text{ m}\mu$  (maximum of metmyoglobin) could be observed. The solutions were prepared by dissolving approximately 6 mg. of the crystals in 5 ml. of  $M/15$  phosphate buffer, pH 7, and the concentration of myoglobin was determined by estimating the iron content of the solution after the readings. The concentration of myoglobin was 1.362 mg./ml. in the heart sample and 1.233 mg./ml. in the urine sample.

The results, reduced to a common concentration of 1 g./l., are represented graphically in Fig. 5. Except for a shift of 2  $m\mu$  toward the blue of the maximum of the  $\beta$ -band of the urine myoglobin, the two curves coincide perfectly. It seems unlikely, however, that this shift may be due to some structural difference between the two samples.

Other more important differences exist between the results we have obtained with human carboxymyoglobin and those obtained with horse CO-myoglobin; the  $\beta$ -band is higher than the  $\alpha$ -band (slightly lower in horse myoglobin); the maximum of the  $\alpha$ -band is at 577  $m\mu$

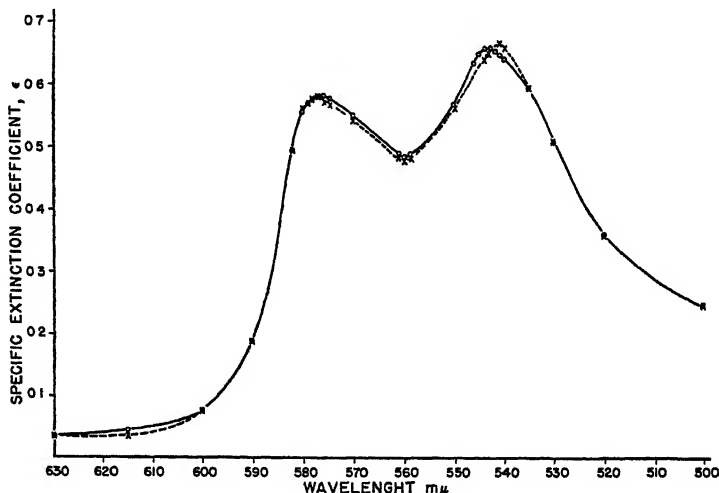


Fig. 4. Light-Absorption Curves of 0.1% Solutions of the CO-Compounds.

● — ● Heart-myoglobin; X - - - - X Urine-myoglobin.

(579 in horse myoglobin) and has an extinction coefficient of only 0.58 (0.73 in horse myoglobin). Although the apparatus used has been periodically calibrated with a mercury lamp and further checked with reduced cytochrome c before and after the readings were taken, we do not wish to comment on these discrepancies before a more thorough investigation has been carried out.

#### *E. Cataphoresis Analysis*

As a further check of purity, a solution of recrystallized heart myoglobin was examined at pH 7.04 in the cataphoresis apparatus of Tiselius. No boundary other than that of myoglobin was observed.

*F. Solubility*

No investigation of the solubility of the purified samples was made. In the crude extracts of heart and urine, myoglobin remained in solution in 75% ammonium sulfate, in which hemoglobin is almost entirely precipitated, and could be salted out by saturation with ammonium sulfate. However, whereas the urine myoglobin was entirely precipitated in this way, a small part remained dissolved in the similarly treated heart extract. In view of the difference in composition of the mother-liquors, no particular significance can be attached to this difference.

## DISCUSSION

The data provided show unmistakably that genuine myoglobin was crystallized both from human heart and urine. The small discrepancies in the crystal shape, light absorption and solubility of the heart and urine samples do not appear sufficiently significant to warrant a conclusion that an essential difference exists between them. Whatever the cause of myoglobinuria, it does not appear to lie in a particular defect of the molecule of myoglobin itself. As might be expected, human myoglobin shows specific properties which distinguish it from the myoglobins of other animal species. Whether the spectral differences are as pronounced as the results obtained thus far appear to show, remains a matter for further investigation.

## SUMMARY

Crystalline human myoglobin with an iron content of 0.34% has been prepared from heart muscle and from myoglobin-containing urine. Only slight, and probably insignificant, differences have been found between the two samples.

Human myoglobin crystallizes in the same fan-shaped clusters of needles as horse myoglobin, but its carboxy compound appears to show some spectral peculiarities as compared with horse myoglobin. This aspect of the problem will be further investigated.

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*Addendum to the proof:* Drabkin, *Am. J. Med. Sci.* **209**, 269 (1945), recently mentions having crystallized human myoglobin from cardiac muscle. The only information given in this paper is that the crystals are probably orthorhombic.

# Activity of Estrone as a Lipotropic Factor

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## INTRODUCTION

In previous communications from these laboratories (1, 2) the use of estrone pellets implanted in the spleen of castrated female rats as a means of assay of specific nutritional hepatic injury of the cirrhotic type has been described. In the course of these experiments it was noted (2, 3) that the incidence and degree of cirrhosis produced was less than that usually observed. This result could best be explained as an effect of the estrone itself on the liver. Since lack of lipotropic factors is a common etiological bond between experimental cirrhosis and fatty liver in rats, the present study was undertaken to see whether estrone would show a protective action against accumulation of fat in the liver.

## EXPERIMENTAL

Young adult female rats of Wistar and Sprague-Dawley stock, most of them weighing between 150 and 200 g., were used in the experiments.

Several series of experiments were conducted. Within each series, when all of the animals could not be run simultaneously, equal numbers of the various groups were run together.

The diet given all animals was one we have used for some years to produce cirrhosis: casein, SMACO, 8; sucrose, 48; Crisco,<sup>1</sup> 40; salt mixture No. 2 (U.S.P. XII), 4.

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<sup>1</sup> With the shortage of fats a particular brand was not always obtainable and "Spry" was occasionally substituted for "Crisco."

Each animal received daily 20  $\gamma$  thiamine chloride, 100  $\gamma$  calcium pantothenate, 20  $\gamma$  pyridoxine and 25  $\gamma$  riboflavin dissolved in 1 ml. of water. Three drops of percomorph oil furnishing 3750 units of vitamin A and 540 units of vitamin D and 3 mg. of  $\alpha$ -tocopherol were given weekly.

The estrone was given orally in all cases: 30  $\gamma$  daily. Crystalline estrone was dissolved in ether, then in cottonseed oil, the ether being evaporated off. The daily dose was contained in 2 drops of oil.

The experimental period was 21 days. The rats were weighed weekly. Food intakes were recorded. At the end of the experiment the animals were etherized, the livers removed and either analyzed directly or kept frozen until the analysis was made. Total lipid was determined essentially according to the method of Tucker and Eckstein (4).

As a check on the findings, the main experiments were carried out in both the Philadelphia and Cleveland laboratories. The results have been tabulated separately in Tables I and II. All figures in the tables are the averages for the group.

TABLE I  
*The Effect of Estrone on the Development of Fatty Livers in  
Intact and Castrated Female Rats \**

Expt.	Treatment	No. of rats	Weight of animals		Food intake	Liver	
			Initial	Change		Weight	Total lipid
I	Intact females		<i>g.</i>	<i>per cent</i>	<i>g/day</i>	<i>g.</i>	<i>per cent</i>
	Cystine	13	166 (150-203)	+5.6 $\pm$ 1.4	6.3 $\pm$ 0.62	9.5 $\pm$ 1.02	22.5 $\pm$ 2.3**
	Cystine + estrone	15	187 (145-205)	-2.4 $\pm$ 1.9	5.3 $\pm$ 0.32	9.3 $\pm$ 0.53	16.2 $\pm$ 2.4
II	Castrate females						
	Control	26	183 (160-206)	-6.0 $\pm$ 1.3	7.4 $\pm$ 0.24	7.9 $\pm$ 0.25	17.6 $\pm$ 1.7
	Estrone	25	181 (150-214)	-10.0 $\pm$ 0.9	6.6 $\pm$ 0.24	7.2 $\pm$ 0.22	13.1 $\pm$ 1.4
	Methionine	25	177 (150-207)	+1.1 $\pm$ 0.9	7.1 $\pm$ 0.16	7.6 $\pm$ 0.24	12.0 $\pm$ 1.0
	Estrone + methionine	26	174 (148-198)	-4.1 $\pm$ 1.0	6.5 $\pm$ 0.24	7.1 $\pm$ 0.16	7.1 $\pm$ 0.36
III	Intact females						
	Methionine	16	159 (145-174)	-2.3 $\pm$ 1.3	6.1 $\pm$ 0.26	7.1 $\pm$ 0.31	9.2 $\pm$ 0.50
	Methionine + estrone	14	161 (147-189)	-5.8 $\pm$ 1.3	6.4 $\pm$ 0.25	7.3 $\pm$ 0.26	6.7 $\pm$ 0.39
IV	Intact females						
	Control	10	154 (141-165)	-7.4 $\pm$ 1.4	5.7 $\pm$ 0.24	6.8 $\pm$ 0.24	14.5 $\pm$ 1.9
	Estrone	11	152 (144-160)	-10.8 $\pm$ 1.1	5.1 $\pm$ 0.26	5.6 $\pm$ 0.17	7.3 $\pm$ 0.51
	Methionine	8	150 (140-159)	-5.2 $\pm$ 1.3	4.9 $\pm$ 0.09	5.8 $\pm$ 0.22	8.0 $\pm$ 0.83

\* Philadelphia Laboratory.

\*\* Standard error of the mean.

TABLE II

*The Effect of Estrone on the Development of Fatty Livers in Intact and Castrated Female Rats \**

Expt.	Treatment	No. of rats	Weight of animals		Food intake	Liver	
			Initial	Change		Weight	Total lipid
			<i>g.</i>	<i>per cent</i>	<i>g/day</i>	<i>g.</i>	<i>per cent</i>
II	Castrate females						
	Control	14	186 (172-206)	+1 ±1.3	8.7±0.2	11.6±0.28	19.3±2.0 <sup>**</sup>
	Estrone	12	197 (176-228)	-13 ±1.0	6.5±0.3	10.4±0.35	15.6±1.7
	Methionine	12	181 (156-202)	0 ±1.4	7.8±0.2	10.9±0.27	11.0±0.9
	Estrone + methionine	10	186 (156-220)	-9 ±2.4	5.9±0.3	9.6±0.30	6.8±0.2
IV	Intact females						
	Control	5	205 (184-226)	+4.1±0.8	7.5±0.2	9.1± .28	15.5±3.3
	Estrone	5	192 (146-231)	-3.4±6.1	6.6±0.9	8.1± .45	11.8±2.0

\* Cleveland Laboratory.

\*\* Standard error of the mean.

In the first experiment intact female rats were used. These animals received 50 mg. of cystine daily (as the sodium salt dissolved in 1 ml. water) to aggravate the antilipotropic effect of the diet. This supplement was not used in the later experiments. As shown in Table I, Exp. I, the level of liver fat was lower in the animals receiving estrone than in the controls, but the difference, although suggestive in that it supported the earlier observations, was hardly large enough to be of statistical significance in view of the rather wide range of values.

To minimize the effect of the rats' own production of estrone and to parallel more closely the earlier experiments with cirrhosis, castrated animals were used in the second experiment. The operation was performed when the rats weighed about 120 g. Generally they were started on the experiment about 3 weeks after castration and in no case did the time exceed 6 weeks.

In this series, in addition to estrone, the effect of methionine and of methionine in conjunction with estrone was studied. The methionine was given as a separate supplement to the diet: 50 mg. per day dissolved as the sodium salt in 1 ml. of water and mixed with the vitamin B supplement. In a few cases, where the animals showed considerable distaste for the methionine, it was given by stomach tube.



Experiment II in Tables I and II show the average values found. The results from the two laboratories parallel each other closely. In this experiment, as in the preceding one, the effect of estrone alone is of doubtful significance, although in both cases the animals receiving estrone had a lower level of liver fat than did the controls. Methionine, as would be expected, showed a definite lipotropic effect, the difference between the average of the controls and the methionine-treated animals being respectively 2.8 and 3.8 times the standard error of the difference of the means<sup>2</sup> in the two experiments. However, the values found, 12.0 and 11.0%, are still definitely elevated. When estrone was used with methionine the results were striking. The liver fat was practically normal (7.1 and 6.8%). Considered statistically, the differences between the averages of the groups with methionine and those with methionine and estrone are 4.7 times as great as their standard error.

Experiments III and IV are a repetition of Experiment II using intact animals. It was of interest to determine whether methionine and estrone together would be as much more effective than methionine alone with unoperated as with operated animals. Also, since, in the preliminary work on the effect of estrone on intact animals, cystine had been used, it was desirable to repeat this experiment without cystine so that the results would be comparable with those on the castrated animals. Since this experiment was not run simultaneously with Experiment III a third group receiving methionine was included.

In general, the levels of liver fat on the intact animals are a little lower than those on the castrates, but since the experiments were not run simultaneously it would be ill-advised to attach too much importance to this observation. Again, it is evident (Experiment III, Table I) that, while methionine protects quite well against development of fatty liver with an average total lipid of 9.2%, estrone with the methionine gives highly significant further protection with an almost normal figure of 6.7%. When the effect of the estrone alone is considered in the small group from the Cleveland laboratory (Table II, Experiment IV), although the average of the estrone-treated animals is lower than that of the controls, there is no significant difference between the two groups. In the corresponding groups from the Philadelphia laboratory (Table I, Experiment IV) the difference is much

<sup>2</sup> A difference of means greater than 2.5 times the standard error of the difference of the means is generally accepted as significant, corresponding to a *p* value equal to or less than .01.

greater, showing a statistically significant variation (3.7 standard differences). In this last experiment the livers of the estrone-treated animals have a lower fat content than those given methionine.

There is correlation between the size and the degree of fat infiltration of the livers but the differences between the various groups are not large. There was a decided difference between the two laboratories in respect to liver size, which may be due to a difference in the strains of animals used.

## DISCUSSION

These experiments indicate that estrone exerts a lipotropic effect. When estrone was used alone the results of all but one of the individual experiments were of doubtful significance but the fact that in every experiment the difference was in the same direction makes it highly improbable that the result was fortuitous. When estrone was used with methionine there was no doubt that it greatly increased the lipotropic effect.

The present experiments substantiate the results with cirrhosis referred to previously. There have been other observations on the influence of sex on pathological changes in liver and kidney. György, Seifter, Tomarelli and Goldblatt (5) reported that, on exposure to carbon tetrachloride, female rats showed better survival rate and less hepatic and renal injury than males. Weichselbaum (6) found female rats less susceptible than males to a characteristic syndrome due to cystine deficiency. Griffith (7) found that renal lesions as a result of choline deficiency appeared more slowly and were less severe in young female rats than in males of the same age and weight. It is well known in human clinical practice that cirrhosis is found much more frequently in men than in women. There are indications that estrogens are linked with fat metabolism. This has been clearly demonstrated in fowls. Entenman, Lorenz and Chaikoff (8, 9) have shown that injections of estrogens will produce an extreme lipemia in both male and female birds, all fatty constituents being affected. Zondek and Marx (10, 11) confirmed these experiments with cocks and called attention to the fattiness of all the organs. They were unable to demonstrate any effect in mammals. Loeb (12) using rats on a high fat diet (71% hydrogenated coconut oil) was unable to show any effect of 300 mg. of estradiol benzoate given in large doses over a short-time interval, but when as

little as 5 mg./day were given for 4 weeks a moderate lipemia was produced. In the course of extensive studies on the toxicology of natural and synthetic estrogens (13-22) divergent conclusions were reached with regard to their effect on the liver. It appears that, in general, estrogens, when used in not excessive doses, were without any consistent effect on the fat content of the liver in rats or dogs kept on a normal laboratory diet. In contrast, an increase of the hepatic glycogen was demonstrated following the administration of estrogenic substances to rats (20, 21, 23, 34).

The lipotropic effect of estrone could be due to a direct effect in the liver. This is not, however, the only possibility. Administered by mouth the hormone is carried in the circulation. That it is not immediately completely inactivated by the liver is shown by the fact that the castrated animals were in estrus throughout the experimental period. It is well known that estrogens affect the general condition of the animal. Spencer, Gustavson and d'Amour (27) showed that injection of 20 units of estrin/day caused a decrease in growth rate, a result attributed to suppression of the growth-promoting hormone of the anterior pituitary. Zondek (28) cured dwarfing resulting from treatment with estrogenic hormone by administration of the pituitary growth factor.

In our experiments, the animals receiving estrone generally ate somewhat less and lost more weight than their controls. The obvious possibility of explaining the differences in the level of liver fat on the basis of differences in food intake was not borne out on examination of the data. In the large series of castrate animals (Experiment II, Table I), although there was an appreciable difference in the intakes of the groups, when all of the animals were considered, 16 animals could be selected from each group which had the same range of intake and the same mean intake. When the liver fat values of these animals were averaged the results differed only slightly from the averages of the complete groups.

The weight changes in the experiments were not large, but there were significant differences between the groups because of the inhibitory effect of estrone and the stimulating effect of methionine. Treadwell, Tidwell and Gast (29) and Treadwell (30) have shown that an animal gaining weight will have a higher percentage of fat in the liver than one which is not, because, in the former case, methionine is used for growth which in the latter is available for lipotropic activity. In

most of their experiments there were very large gains in weight. In the present experiments the majority of the animals were losing weight. The only group showing an average gain in weight were the castrated animals treated with methionine. It might seem probable that the liver fat is higher in this case than when estrone is used with the methionine because some of the methionine is diverted from its function as a lipotropic agent. Equally, the large loss in weight in some of the groups might make available to the liver methionine from the body tissue. In the experiment on castrate rats where the groups were large enough to justify such treatment (Experiment II, Table I), an attempt was made to evaluate this factor. Table III shows the values

TABLE III

*Relation of Total Lipid to Weight Change in Castrate Female Rats*

Group	Number of rats	Weight change	Total lipid
		<i>per cent</i>	<i>per cent</i>
Control	13	- 11.4(- 18.3 to - 6.8)	19.1
	13	- 1.2(- 6.3 to + 10.2)	16.1
Estrone	12	- 13.7(- 19.2 to - 9.1)	13.6
	13	- 6.5(- 8.6 to + 2.2)	12.6
Methionine	13	- 2.6(- 7.1 to - 0.6)	9.5
	12	+ 5.2(+ 0.7 to + 9.4)	14.6
Estrone+methionine	13	- 8.2(- 12.4 to - 5.9)	7.4
	13	- 0.4(- 5.1 to + 6.8)	6.9

found if each group is divided in half on the basis of weight change. In three of the groups the difference in liver fat is negligible and, in fact, in a direction opposite to that which might have been expected. In the methionine group those animals which gained weight show a liver fat enough higher than those which lost to suggest that weight change does play a role. However, there is still a significant difference between the animals receiving methionine which lost weight and that portion of the methionine-estrone group which had about the same weight change. When 11 animals from the methionine group were matched with 11 from the methionine-estrone group which showed identical weight changes average liver fats of 12.3 and 6.9% were ob-

tained: the same values as for the whole group in each case. Thus, though estrone may act in part through its effect on growth this is by no means a complete explanation of its lipotropic activity.

At the present stage no explanation can be offered for the observed action of estrone. Comparable experiments must be made with males. There have not been reports in the literature of sex differences in the production of fatty livers. The amount of estrone used in these experiments was large but not excessive.

We are greatly indebted to Miss Ethel Buchwald for technical assistance.

### SUMMARY

Estrone shows a small but definite lipotropic effect when fed at a level of 30  $\gamma$ /day to intact and castrated female rats on a diet which produces fatty livers. When estrone is used with methionine it augments greatly the lipotropic action of the latter and essentially normal liver fat values are obtained. No explanation has yet been found for this action by estrone.

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# Effect of Xanthophylls, Chlorophylls, Sulfasuxidine and $\alpha$ -Tocopherol on the Utilization of Carotene by Rats

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## INTRODUCTION

Previous papers from this laboratory (3, 7) reported that the carotene of vegetables is utilized to a lesser extent than carotene dissolved in cottonseed oil for storage of vitamin A in livers of rats. Possibly the utilization of carotene in vegetables is affected by substances that accompany it in plants. Woolley and White (8) have reported that the pyridine analogue of thiamine appreciably reduces the biological activity of thiamine. Xanthophylls being similar to the carotenes in chemical structure may affect the utilization of carotenes. Pepkowitz (6) found that chlorophyll destroyed carotene dissolved in petroleum ether when the solution was exposed to light. Harris, Kaley and Hickman (5) have reported that tocopherols preserve carotene in the intestinal tracts of rats. Guggenheim (4) suggested that the utilization of carotene in plants for liver storage of vitamin A was dependent upon the vitamin E content of the plant. Previous work in this laboratory (3) showed that the addition of vitamin E to plant materials did not increase the storage of vitamin A in the liver.

That sulfonamides depress the bacterial synthesis of the B complex vitamins is well known (1). Whether or not they have any effect on the bacterial destruction of carotene in the intestinal tract has not been ascertained.

The experiments reported in this paper were devised to determine the effect of xanthophylls, chlorophylls and sulfasuxidine on the utilization of carotene for liver storage of vitamin A and to determine

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the effect of  $\alpha$ -tocopherol when fed at higher levels than in our previous report (3).

## EXPERIMENTAL

*Treatment of Animals.* In all the experiments, rats approximately 28 days old and reared by females fed on a diet low in vitamin A were used, and for each experiment two groups of 6 rats each, paired as to sex and litter. The basal ration contained white corn meal 59%, casein 22%, yeast 9%, irradiated yeast 1%, cottonseed oil 4%, salt mixture 4% and sodium chloride 1%. The rats were fed their respective test diets for 14 days and were killed on the 15th day.

The storage of vitamin A in the rat livers was measured by means of a Bausch and Lomb medium quartz spectrograph by a method already described (2).

*Xanthophylls.* The mixture of xanthophylls was prepared by chopping with cold alcoholic potassium hydroxide in a Waring Blendor an amount of fresh spinach equivalent to 9000  $\gamma$  of carotene. The chopped material was extracted with alcohol and petroleum ether until all the yellow pigment was removed. An equal volume of water was added to the extract and the carotenes removed by shaking with petroleum ether. The petroleum ether solution was washed with 90% methanol to recover any xanthophylls and the washings combined with the alcohol-water solution. The alcohol-water solution was diluted and the xanthophylls extracted with ethyl ether. The ethyl ether solution was dried over anhydrous sodium sulfate and evaporated nearly to dryness *in vacuo*. The xanthophylls were taken up in 15 ml. of cottonseed oil which contained 9000  $\gamma$  of purified carotene. One-tenth ml. of this oil solution contained 60  $\gamma$  carotene together with the quantity of xanthophylls which would accompany this amount of carotene in fresh spinach. Each rat in one group received 0.1 ml. of this oil solution and the replicas in the second group 0.1 ml. of oil containing an equal amount of carotene but no xanthophyll.

*Chlorophyll.* Chlorophyll was prepared from the quantity of green spinach which contained 900  $\gamma$  of carotene. The spinach was chopped in a Waring Blendor with methanol, and the methanol drained off. The residue was chopped in the blendor with 9 parts petroleum ether and 1 part benzol, filtered off and washed with the solvent until all the green color was removed. The combined filtrates were washed with water, dried with anhydrous sodium sulfate and chromatographed on powdered sucrose. The carotene and xanthophyll were washed through the column and the chlorophyll was eluted with petroleum ether containing 2% ethanol. The alcohol was removed by shaking with water, the petroleum ether dried with sodium sulfate and the chlorophyll again chromatographed on powdered sucrose. The chlorophyll was eluted, the solvent evaporated and the chlorophyll taken up in 15 ml. of cottonseed oil containing 9000  $\gamma$  of purified carotene. One-tenth ml. of this solution contained 60  $\gamma$  of carotene and the quantity of chlorophyll which accompanied this amount of carotene in the fresh spinach. The rats were fed as in the experiments on xanthophylls.

*Tocopherol.* Each rat in one group was fed daily 0.5 mg.  $\alpha$ -tocopherol in 0.1 cm. lard and 60  $\gamma$  carotene as spinach, and each replica in the second group received the spinach only.

*Sulfasuxidine.* Each rat in one group received daily 60  $\gamma$  of carotene as spinach, and the basal diet into which had been incorporated 1% sulfasuxidine. The replicas in the

second group were treated in the same manner except that no sulfasuxidine was added to the basal diet.

### DISCUSSION OF RESULTS

The results are given in Table I. Xanthophylls and chlorophylls when fed with carotene decreased the utilization of carotene for liver storage of vitamin A about 20%. This may account in part for the low utilization of carotene in many vegetables.

TABLE I

*Effect of Xanthophylls, Chlorophyll, Sulfasuxidine and  $\alpha$ -Tocopherol upon Storage of Vitamin A in Livers*

	Weight of liver	Spectro-vitamin A in liver	Spectro-vitamin A in liver
	g.	$\gamma/g.$	$\gamma/liver$
Carotene in oil	4.0	19.2	77
Carotene in oil with xanthophyll	3.6	16.8	59
Carotene in oil	4.5	18.3	82
Carotene in oil with xanthophyll	4.1	15.2	62
Carotene in oil	3.6	21.0	76
Carotene in oil with xanthophyll	3.6	17.4	63
Carotene in oil	4.6	22.1	92
Carotene in oil with chlorophyll	4.3	17.5	75
Spinach	4.4	5.7	25
Spinach with sulfasuxidine	3.8	6.8	26
Spinach	6.8	5.6	38
Spinach with sulfasuxidine	6.1	6.0	37
Spinach	4.2	7.8	33
Spinach with $\alpha$ -tocopherol	4.3	8.4	36
Spinach	4.6	8.1	37
Spinach with $\alpha$ -tocopherol	4.9	6.8	33
Spinach	4.9	6.7	33
Spinach with $\alpha$ -tocopherol	4.4	6.8	30

Tocopherol when fed with spinach did not increase the utilization of carotene for liver storage of vitamin A. This finding is not necessarily in disagreement with the suggestion of Guggenheim (4) that the utilization of carotene depended upon the vitamin E content of the plant. In this paper we report the effects of  $\alpha$ -tocopherol added to plant material and Guggenheim correlated the percentage of carotene utilized with the amount of tocopherols in the plant tissue.

Sulfasuxidine did not affect the utilization of carotene for liver storage of vitamin A. It is apparent that sulfonamide does not reduce the activity of any carotene-destroying bacteria in the intestines of rats.

### SUMMARY

Xanthophylls and chlorophylls when fed with carotene dissolved in cottonseed oil decreased the utilization of carotene for liver storage of vitamin A about 20%. Alpha-tocopherol or sulfasuxidine did not affect the utilization of carotene in spinach for liver storage of vitamin A.

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# The Metabolic Conversion of Tryptophan to Nicotinic Acid and to N'-Methylnicotinamide \*

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## INTRODUCTION

Voegtlin (1), as early as 1914, postulated that a deficiency of certain amino acids in the diet may be a factor in the incidence of pellagra. In 1915, Goldberger and associates (2) showed that pellagra could be prevented when the diet was supplemented with high protein foods such as meat, eggs and dried beans. More recently it has been shown by Wintrobe *et al.* (3) that the level of protein in the diet is a definite factor in the requirements of pigs for nicotinic acid. The usual symptoms of nicotinic acid deficiency were readily produced when a diet containing 10% of casein was fed as the only source of protein, whereas on diets containing 26.1% of casein there were no signs of nutritional deficiency except for slightly less satisfactory growth in some of the pigs.

Recent studies with laboratory animals have shown that an interrelationship exists between tryptophan and nicotinic acid in the nutrition of the albino rat (4, 5, 6). Specifically, it appears that tryptophan can be utilized by this animal for the synthesis of nicotinic acid (5, 6).

In the present work a study of this relationship was extended to 2 other species—the horse, which apparently does not require a dietary source of nicotinic acid (7) and the cotton rat (*Sigmondon hispidus hispidus*), which requires a dietary source of nicotinic acid (8). The effect of feeding different amounts of tryptophan and nicotinic acid on the urinary excretion of nicotinic acid, N'-methylnicotinamide and tryptophan is reported in this paper.

## EXPERIMENTAL

The horses used in these studies were Shetland ponies divided into 2 groups with 3 animals in each group. The maintenance and dietary regimen of the horses were

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\* We are indebted to the Dow Chemical Company for some of the *dl*-tryptophan and to Merck & Company for the nicotinic acid used in these investigations.

essentially as described previously (7). The basal diet was made up of dried beet pulp, corn, casein, salts and vitamin supplements and provided approximately 9.0 mg. of nicotinic acid and 3 g. of tryptophan/day. The tryptophan content of the basal diet was calculated from the data of Block and Bolling (10).

One group received 6 g. of *dl*-tryptophan/day for 3 days, while the other group received 3 g. of *dl*-tryptophan daily for 3 days. Equal quantities of the basal ration were fed twice daily. When supplements were given, they were added to one feeding and this portion of feed was usually consumed within 2 to 3 hours. Urine collections were made prior to tryptophan feeding, the second and third days of supplementation and on the two days after the feeding of tryptophan was discontinued. In some cases further collections were made 5 days later.

In all cases the collections were analyzed for the amount of *N'*-methylnicotinamide, nicotinic acid and tryptophan. *N'*-methylnicotinamide was determined fluorometrically (9, 11) and nicotinic acid was determined microbiologically (12) without hydrolysis of the samples. Both of these methods have been used for similar studies reported previously (9). The apparent free tryptophan was determined in urine and also in blood microbiologically as described in previous work (13, 14, 15).

The reverse process was also studied, namely, the possible conversion of nicotinic acid to tryptophan. For these experiments 4 g. of nicotinic acid were fed to 2 horses and the amount of tryptophan excreted was measured.

Cotton rats 75-125 g. in weight were used in the present study. A finely ground basal diet\* was fed and urine collections made for a period of 3 days. Toluene and HCl were added as preservatives to the receiving flasks. One hundred mg. of *dl*-tryptophan were fed/rat/day in addition to the basal diet for a period of 3 days. Total urine collections were made during this period and for a similar period after feeding. The food intake for the 2 groups of cotton rats was restricted slightly, so that all the basal diet and supplement would be consumed within 24 hours. The amounts of nicotinic acid and *N'*-methylnicotinamide excreted were measured. The influence of nicotinic acid ingestion (50 mg./rat/day) on the excretion of *N'*-methylnicotinamide was also studied.

## RESULTS AND DISCUSSION

It is readily apparent (Table I) that the ingestion of 6 g. of *dl*-tryptophan in addition to that in the basal diet resulted in an approximately 3-fold increase in the amount of nicotinic acid excreted by the horse. The variation in the values from day to day for individual animals and between different animals is due, in part, to the volume of urine voided, as the variation is appreciably less when the values are calculated on a basis of 100 ml. The volume of urine voided during a 24-hour period varied from 0.7 l. to 4 l. Little increase could be demonstrated when only 3 g. of *dl*-tryptophan were fed/day. The amount of tryptophan excreted was also increased when added tryptophan was

\* Purina dog chow.

TABLE I

*Effect of the Ingestion of Tryptophan on the Excretion of Nicotinic Acid,  
N'-methylnicotinamide and Tryptophan by the Horse*  
(Values expressed as mg. excreted/24 hours)

Dietary regimen	Collection days	Animal No.	Nicotinic acid	N'-methyl-nicotinamide	Tryp- to- phan
Basal	1-2	1	1.4	3.0	39.5
		2	2.0	4.1	26.2
		3	1.9	3.7	31.0
	Average		1.8	3.6	32.2
Basal plus 6 g. of <i>dl</i> -tryptophan/day (fed on days 3, 4 and 5)	4-6	1	5.5	3.0	59.0
		2	7.5	5.5	90.0
		3	7.8	4.8	82.0
	Average		6.9	4.4	77.0
Basal	7	1	3.4	9.9	38.4
		2	6.8	4.6	31.6
		3	2.9	3.2	35.0
	Average		4.4	5.9	35.0
Basal	12	2	3.1	1.8	35.3
		3	4.0	3.2	25.8
Basal	1-2	4	1.6	3.9	32.6
		5	2.5	3.8	49.5
		6	4.2	11.1	56.5
	Average		2.8	6.3	46.2
Basal plus 3 g. of <i>dl</i> -tryptophan/day (fed on days 3, 4 and 5)	4-6	4	3.2	5.8	58.0
		5	3.6	5.1	86.0
		6	3.5	5.8	92.0
	Average		3.4	5.6	79.0
Basal	7	4	1.8	3.8	36.0
		5	1.8	4.4	46.5
		6	2.4	5.6	48.0
	Average		2.0	4.6	43.5

fed. The fact that the ingestion of tryptophan did not result in an increase in the excretion of *N'*-methylnicotinamide was to be expected since it had been previously shown that this compound is not the major end product of nicotinic acid metabolism in the horse (9).

Other workers (5) have reported an approximately 10-fold increase in the amounts of nicotinic acid and N'-methylnicotinamide excreted when 50 mg. of *l*-tryptophan were fed/day to albino rats receiving a 15% casein basal ration. This added amount of tryptophan was approximately 2.5 times that contained in 15% casein (1.3% tryptophan (16)). In the work with the horse the highest level of tryptophan added was equal to the amount in the basal diet (assuming the *d*-isomer to be inactive, see Rosen *et al.* (5)). Therefore, the results are of the same order for the white rat and horse in the case of nicotinic acid excretion. A difference was, however, observed in the case of N'-methylnicotinamide. The horse does not excrete additional quantities of this compound while the white rat does when added tryptophan is fed.

The data obtained on the excretion of apparent free tryptophan show that only a small percentage of the tryptophan ingested is excreted (approximately 1%). This amount is similar to that observed for the white rat (15). The amount excreted varied with the dietary intake, which is in agreement with results observed with the mouse and white rat (15). In some cases, the amount of apparent free tryptophan in the blood serum was measured 3-4 hours after feeding tryptophan and approximately a 3.5-fold increase in the concentration was noted (12.0  $\gamma$ /ml. when the basal diet was fed and 41.6  $\gamma$ /ml. when 6 g. of *dl*-tryptophan were fed). The concentration in the blood had decreased to the level on the basal diet within 24 hours after feeding the tryptophan. Thus, it appears that the added tryptophan was rapidly metabolized and only small amounts were excreted.

The conversion to nicotinic acid is very rapid, as an increase in the amount excreted is noted within 24 hours after feeding, and this level is maintained for only about 24 hours after tryptophan feeding has been discontinued. Thus, the figures in Table I are combined for the first 3 collections after the basal period. This includes 2 collections during the period the tryptophan was fed and one the following day. The decreased excretion of both nicotinic acid and tryptophan, after tryptophan supplementation was discontinued, is apparent in Table I for collection days 7 and 12.

The amount of tryptophan excreted when 4 g. of nicotinic acid were fed was the same as when the animals were fed the basal diet (34.5 and 32.0 mg./day, respectively) thus affording evidence that, at least in the horse, nicotinic acid is not converted to tryptophan.

The comparative studies conducted with the cotton rat indicate that this species, like the horse and the albino rat, can convert tryptophan into nicotinic acid. Unlike the horse the cotton rat appears to convert nicotinic acid to *N'*-methylnicotinamide, as does the albino rat (17) and human (18). When 100 mg. of *DL*-tryptophan were added daily to the basal diet of the cotton rat there was a marked increase in the excretion of *N'*-methylnicotinamide (Table II). The magnitude of these

TABLE II

*Effect of the Ingestion of Tryptophan and Nicotinic Acid on the Excretion of N'-methylnicotinamide by the Cotton Rat*  
(Values expressed as  $\gamma$  excreted/rat/day)

Dietary regimen	Collection days	N'-methylnicotinamide	
		Group 1 (4 rats)	Group 2 (4 rats)
Basal	1-3	167	86
Basal plus 100 mg. of <i>DL</i> -tryptophan	4-6	518	282
Basal	7-9	162	203
Basal	10-12	174	97
Basal plus 50 mg. of nicotinic acid	13-15	1622	894
Basal	16-18	703	372
Basal	19-21	—	124

values was not as great as for the white rat (5). The *N'*-methylnicotinamide values decreased to the values on the basal diet within a relatively short time when the feeding of tryptophan was discontinued. The amount of *N'*-methylnicotinamide excreted when the basal diet was fed varied for the two groups. The rats in group I were larger and their daily food intake was about 9 g./day as compared with 6 g. for group 2. Little effect was noted on the amount of nicotinic acid excreted and there was considerable variability in these values.

To obtain more information on the end products of nicotinic acid metabolism in the cotton rat, *N'*-methylnicotinamide was measured in the urine when nicotinic acid was fed. A 10-fold increase in the excretion of *N'*-methylnicotinamide was noted when 50 mg. of nicotinic acid/day were added to the basal diet, thus indicating that this is one of the chief end-products of nicotinic acid metabolism in the cotton rat.



The amount of nicotinic acid excreted was not measured since spillage of food might invalidate the results.

The data obtained indicate an interesting species difference in the metabolic conversion of dietary tryptophan to nicotinic acid or its derivatives. The cotton rat, which requires a dietary source of nicotinic acid, excretes considerable quantities of the methylated derivative (N'-methylnicotinamide) when tryptophan is fed. The horse excretes more nicotinic acid but not N'-methylnicotinamide, while an increase in the excretion of both compounds occurs when the albino rat is fed added tryptophan (5). Further studies will aid in elucidating the pathways by which these transformations occur.

The data on the albino rat, the cotton rat and the horse, showing that tryptophan may serve as a precursor of nicotinic acid, afford an explanation of earlier observations that the usual symptoms of pellagra or a nicotinic acid deficiency are rarely encountered on high protein diets (1, 2, 3).

#### SUMMARY

1. The effect of the ingestion of added tryptophan on the excretion in the urine of nicotinic acid, N'-methylnicotinamide and tryptophan by the horse and cotton rat was investigated.

2. When 6 g. of *dl*-tryptophan were fed to horses in addition to the basal diet a 2 to 4-fold increase occurred in the amount of nicotinic acid excreted. There was no appreciable effect on the amount of N'-methylnicotinamide excreted.

3. Tryptophan also appears to serve as a precursor of nicotinic acid for the cotton rat in that, when tryptophan or nicotinic acid was fed, there was a significant increase in the amount of N'-methylnicotinamide excreted.

4. While the ingestion of tryptophan resulted in a significant increase in the amount of free tryptophan in the blood and urine of the horse only about 1% of the added tryptophan could be accounted for in the urine.

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# The Production of Methionine Deficiency in the Rat with Low Casein Diets

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## INTRODUCTION

In their studies of the nutritive value of proteins in the growth of the rat, Osborne and Mendel (1) found that, with a diet containing 9% of casein as the protein, growth was promptly limited by the protein factor. The addition of isolated cystine at once rendered the ration decidedly more adequate for growth. Jackson and Block (2) obtained a similar stimulation of growth when methionine was added to their basal diet which was poor in cystine. Using mixtures of highly purified amino acids, Womack, Kemmerer and Rose (3) came to the conclusion that methionine and not cystine was the indispensable amino acid although it might be partially replaced in the diet by cystine (4). Diets similar to the 9% casein diet of Osborne and Mendel (1) have been quite generally used to produce methionine deficiency in the rat. The present study was occasioned by our finding that, in using a highly purified 9% casein diet for the production of methionine deficiency, the control rats receiving methionine grew at only a third of the normal growth rate, an observation which we felt needed explanation.

## EXPERIMENTAL AND DISCUSSION

The control diet which occasioned this study is diet 36 in Table I. It will be noted that this diet, which contained 9% of casein and 0.5% of *dl*-methionine, resulted in a growth of 0.9 g./day as compared with 3.0 g./day for a similar diet (diet 31) which contained 20% of casein. Three g./day is also the growth rate of our stock animals over a similar

TABLE I  
*Composition of Diets and Corresponding Rates of Growth*

Diet No.	31	36	38	48	40
	All figures, per 100 g. of diet				
Casein <sup>1</sup>	20.0	9.0	9.00	16.3	9.0 g.
<i>D</i> -Methionine		0.5	0.40	0.2	0.5 g.
Amino acid mixture			1.57		
Sucrose	70.8	81.3	79.83	74.3	
Starch (corn)					81.3 g.
Inositol					2.5 mg.
<i>p</i> -Aminobenzoic acid					2.5 mg.
Nicotinic acid					2.5 mg.
Growth, g./day	3.0	0.9	3.1	3.1	2.7
No. of rats in group	10	10	8	13	8

All the above diets contained salt mixture 4 g., cod liver oil 2 g., cottonseed oil 3 g., choline chloride 0.2 g., calcium pantothenate 2.0 mg., pyridoxine hydrochloride 0.4 mg., thiamine hydrochloride 0.4 mg., riboflavin 1.6 mg. The salt mixture was as used by McKibben, Madden, Black and Elvehjem (5).

age span when fed dog chow supplemented with milk, carrots and lettuce 3 times per week.

All rats used in this study were kept in screen-bottomed cages, usually 1 or 2 rats to a cage, although occasionally as many as 6 were kept in one cage. The rats were fed daily and weighed semiweekly. Wherever possible, when comparisons were to be made between the growth resulting from feeding different diets, litter mates were divided between the groups on the diets. The rats were placed on the diets when 22-32 days of age and the growth figures represent the average growth on the rats in the group during some 7 weeks on the diet in question.

Since the synthetic diet containing 20% casein (diet 31) gave normal growth while the diet containing 9% casein and 0.5% methionine (diet 36) did not, it seemed likely that the limiting factor in the latter diet was some amino acid or acids which were supplied in suboptimal amounts by the diet. The percentage of each of the indispensable amino acids contained in this diet was calculated from the chemical analyses as given by Block and Bolling (6) and from the values obtained by Stokes, Gunness, Dwyer and Caswell (7) with microbiological assays. In Table II the calculated indispensable amino acid

<sup>1</sup> Vitamin-Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE II  
*Indispensible Amino Acids of Diets*  
 (All figures expressed as per cent of the diet)

	As supplied by diet 36		Required by the rat	Additional amino acids added to diet 38 <sup>2</sup>
	Chem.	Microbiol.		
Histidine	0.21	0.24	0.4	0.20
Arginine	0.35	0.34	0.2	
Lysine	0.59	0.66	1.0	0.41
Leucine	1.02	0.85	0.8	
Isoleucine	0.55	0.48	0.5	
Valine	0.59	0.58	0.7	0.15
Methionine	0.30	0.22	0.6	
Threonine	0.33	0.36	0.5	0.20
Tryptophan	0.15	0.10	0.2	0.10
Phenylalanine	0.44	0.51	0.7	

content of diet 36 is compared with the requirement of the rat for these amino acids as given by Rose<sup>3</sup> (8). The calculations are based on the analysis of the casein preparation we used as given by the manufacturer. It may be seen that our 9% casein diet (diet 36), according to both chemical and microbiological assays, appears to be suboptimal with respect to histidine, lysine, valine, threonine and tryptophan. Although the phenylalanine level is low, it was felt that the considerable amount of tyrosine present in casein could probably substitute for a part of the required phenylalanine.

We next prepared a diet (diet 38) which was similar to diet 36 except that corresponding amounts of sucrose had been replaced with the amounts of amino acid as shown in Table II. Since we completed this work, Hodson and Krueger (10) have reported values for the indispensable amino acid content of casein obtained with microbiological assays which differ somewhat from those given by Stokes, Gunness,

<sup>2</sup> To supply these amounts of amino acid in a form effective for growth, the amounts of the compounds given below were added to diet 38:

0.25% of *L*(-)-histidine monohydrochloride, 0.52% of *L*(-)-lysine monohydrochloride, 0.30% of valine, 0.40% of threonine and 0.1% of tryptophan. The last three were racemic amino acids.

<sup>3</sup> The values given in our table include the modifications given by Womack and Kade (9).

Dwyer and Caswell (7). However, diet 38 contained a sufficient amount of the added amino acids to cover the discrepancies. Since the diet to which the 5 additional amino acids had been added (diet 38) resulted in normal growth, it thus seems clear that the poor rate of growth obtained with the use of the 9% casein diet with added methionine (diet 36), was due to the fact that the diet supplied suboptimal amounts of 5 of the indispensable amino acids. As the amount of phenylalanine which we calculated to be present in the diet was only about 5/7 of that required by the rat, it seems likely that the tyrosine substituted for a part of the required phenylalanine. This is in accord with the results obtained by Abderhalden (11).

There still remained to be explained the difference between the low rate of growth which resulted from feeding a 9% casein diet with added methionine and the satisfactory growth obtained by other investigators using similar diets. It seemed likely that this difference could be explained on the basis of intestinal synthesis of the amino acids in question. It is fairly well established that the rat may profit from the biosynthesis of vitamins by the intestinal flora. When rats were placed on a diet suboptimal with respect to riboflavin, Mannering, Orsini and Elvehjem (12) obtained poorer growth and less fecal excretion of riboflavin with sucrose than with starch or dextrin. McIntire, Henderston, Schweigert and Elvehjem (13) found that the growth of rats on a thiamine-poor diet was stimulated by the administration of *p*-aminobenzoic acid and inositol. In the one instance, the dextrin or starch, and in the other, the *p*-aminobenzoic acid and inositol were presumed to favor synthesis by the intestinal flora of the vitamin in question. Our diet 36, which gave suboptimal growth, contained sucrose which had seemed to favor least intestinal synthesis of riboflavin; and the B vitamins were added in pure crystalline form with no inositol or *p*-aminobenzoic acid added. Other investigators who used 9% casein or similar diets have used starch or dextrin as the carbohydrate and have used vitamin B concentrates or other means of adding B vitamins which probably resulted in the inclusion of at least some inositol and *p*-aminobenzoic acid in the diet. It thus seemed likely that, with a 9% casein diet, the small deficiencies in amino acids other than methionine might be made up through intestinal synthesis of the amino acids if the characteristics of the diet were suitable. We, therefore, prepared diet 40 which was a 9% casein diet containing methionine, with starch as the carbohydrate and with added inositol, *p*-amino-

benzoic acid and nicotinic acid, to determine if we could obtain normal growth with such a diet. It will be noted that this diet resulted in essentially normal growth, indicating that such a synthesis apparently did take place. Calculations from the amino acid analyses of casein (6, 7, 10) suggested that about 16.3% of our casein preparation in a diet would be required to supply sufficient amounts of the indispensable amino acids other than methionine. Such a diet (diet 48), with added methionine, resulted in normal growth. Osborne and Mendel (1) found that the minimum amount of casein that the food must contain to promote normal growth in the rat was 18%. Although they also say that with 15% of casein in the food, growth was at a normal rate, they apparently considered 18% of casein to be at least slightly better for growth than 15%.

#### ACKNOWLEDGMENTS

This study was aided by grants from the John and Mary R. Markle Foundation and from Merek and Company. We wish to thank Dr. S. A. Singal for helpful suggestions and Misses Christina Yates and Elizabeth Thompson for the care of the animals.

#### SUMMARY

A 9% casein diet containing added methionine and with sucrose as the carbohydrate, when fed to rats, resulted in growth at about  $\frac{1}{3}$  the normal rate. Calculations from analytical data indicated that this diet was suboptimal with respect to histidine, lysine, valine, threonine and tryptophan. When these amino acids were added to the diet or when the sucrose was replaced by starch, and inositol, *p*-amino-benzoic acid and nicotinic acid were added, normal growth resulted. The significance of these observations is discussed.

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## LETTERS TO THE EDITORS

### On the Localization of the Specific Cholinesterase in Human Blood

From recent investigations (1, 2, 3) it is known that human blood contains 2 different types of cholinesterase (ChE): a specific ChE in the red blood corpuscles which splits choline esters, and a non-specific one in the serum which splits not only choline esters but also non-choline esters. However, information is lacking on the localization of the specific ChE in human blood corpuscles. As will be seen from the following, the specific ChE is bound to the erythrocyte membrane.

The estimation of the ChE activity was made by the manometric method (4). The solution to be examined was adjusted to pH 7.2, and 0.5 ml. of the solution was placed in the main compartment of the Warburg flask. Five-tenths ml. of a 2% acetylcholine chloride solution was placed in the side bulb. The solutions were shaken with 5%  $\text{CO}_2$  in nitrogen for about 10 minutes in a water thermostat at 37°C. When equilibrium was attained, the bulb contents were transferred into the main compartment and the  $\text{CO}_2$  production measured. The activity is expressed in  $\text{mm}^3$ .  $\text{CO}_2$  measured in the first 30 min. when 0.5 ml. sample is used. Correction was made for temperature alterations by using a thermobarometer ( $3.65 \text{ ml. Ringer}_{30} + \text{CO}_2$ ).

The red blood corpuscles from 100 ml. human heparinized whole blood were separated by centrifugation and washed 3 times with isotonic NaCl solution. The cells were hemolyzed by treatment with 500 ml. distilled water. The activity of the hemolyzate was 139, but the activity of the second and third rinses was negligible. The membranes were precipitated from the hemolyzate by adjusting the pH of the solution to about 6.5 with 0.1 *N* HCl (5). After centrifugation the clear supernatant fluid (570 ml.) was decanted. The activity of this fluid was almost zero. The membrane precipitate contained about 30–40% of the hemolyzate activity. When the membranes were washed again with a phosphate buffer at pH 6.5 it was found that the wash water contained no activity; all activity was found in the membranes.

It is obvious that the ChE activity of the erythrocyte is bound to the membrane, and it is so firmly bound that it has hitherto been impossible to elute it to any significant degree.

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### **The Beneficial Effect of Folic Acid (*Lactobacillus casei* Factor) on Lactation in Mice Maintained on Highly Purified Diets**

In a previous report Cerecedo and Vinson (1) showed the beneficial effect of *L. casei* factor on lactation in rats, although the number of animals used was small due to the small quantity of the vitamin available. In the present communication we wish to report the results obtained with mice. An improvement in the lactation performance of mice kept on synthetic rations, when given a concentrate of folic acid, has been observed previously in this laboratory (2).

The diet used was as follows: Purified casein (Smaco), 30; sucrose, 48; Crisco, 10; lard, 5; Osborne and Mendel salts,<sup>1</sup> 5; and Ruffex, 2. To this mixture the following vitamin supplements were added (per kg.): thiamin, 20 mg.; riboflavin, 20 mg.; pyridoxin, 20 mg.; calcium pantothenate, 40 mg.;  $\alpha$ -tocopherol, 20 mg.; vitamin A concentrate, 67.5 mg. (67,500 units), and vitamin D (Drisdol),<sup>2</sup> 5000 units. To this basal diet (R-5a) 10 mg. of synthetic *L. casei* factor was added, to give diet RS-5(10).

The results are summarized in Table I. The beneficial effect of *L. casei* factor may be seen in the fact that of the 23 positive results, 7 were

<sup>1</sup> The amount of  $MnSO_4$  in the salt mixture was doubled.

<sup>2</sup> Obtained from Winthrop Chemical Company, New York.

obtained with third generation RS-5(10) animals. The average weaning weight of the young of these animals was 8.5 g. The beneficial effect of *L. casei* factor may also be seen in the greater gain in weight of the lactating mice as compared with those receiving diet R-5a.

TABLE I

*Effect of L. casei Factor on Lactation in Mice*

Diet	No. of litters born	No. of litters weaned	Average litter size	Average No. of young weaned per litter	Average weaning weight	Average gain in weight of lactating mice
R-5a	29	11	5.3	1.8	g. 8.2	g. 1.0
RS-5(10)	29	23	5.8	4.0	8.2	3.8

This investigation was aided by a grant from the John and Mary R. Markle Foundation. We are indebted to Drs. E. L. R. Stokstad and B. L. Hutchings of the Lederle Laboratories for a generous supply of *L. casei* factor, and to The Borden Company for the vitamin A concentrate.

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October 4, 1946.

## ERRATA

On page 257 of Volume 11, Number 2, the following figure was erroneously omitted:

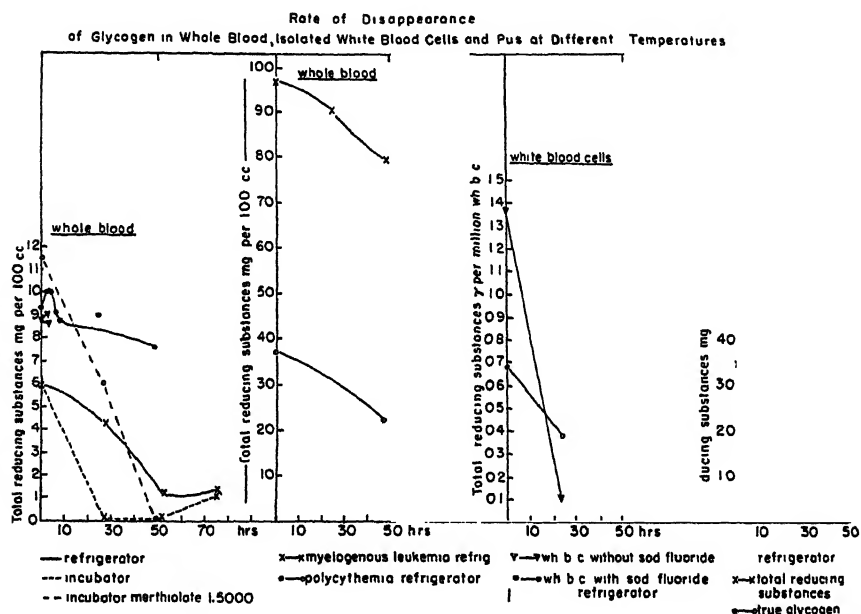


CHART 1.

## Book Reviews

**In Vitro Studien zur Lokalen Sulfanilamid-Therapie.** By JULIUS HIRSCH. Hygiene-Institut der Universität Istanbul. *Compte Rendus Annuel et Archives de la Société Turque des Sciences Physiques et Naturelles* 13, (1945-46). Reprint of 30 pp.

Hirsch gives extensive experimental data on the action of nine sulfa drugs; sulfanilamide, sulfacetamide (*N*<sup>1</sup>-acetyl-sulfanilamide), sulfaguanidine (sulfanilylguanidine), Irgamid (*N*<sup>1</sup>-dimethylacroylsulfanilamide), Irgafen (*N*<sup>1</sup>-3,4-dimethylbenzoylsulfanilamide), sulfapyridine (2-sulfanilamidopyridine), sulfathiazole (2-sulfanilamidothiazole), sulfadiazine (2-sulfanilamidopyrimidine), and Marfanil (*p*-aminomethylbenzenesulfonamide hydrochloride), upon bacteria growing at 37°C., in a medium containing peptone, yeast extract, lactate and mineral salts, and buffered at pH 7.0. His test organisms are *Staphylococcus pyogenes aureus* (5 strains); *Staph. pyogenes albus*, *Streptococcus hemolyticus* and *Escherichia coli* (2 strains of each), and one strain of *E. coli anaerogenes*, *Pseudomonas aeruginosa*, *Eberthella typhosa*, *Bacterium paratyphosum C* (*Salmonella hirschfeldii*) and *Bacterium paracoli*.

His main criterion of the action of the drugs is a depression of respiration of the bacterial cells, measured in a Warburg apparatus. For 0.01 *M* concentrations of a given drug acting upon a given organism, the depression of respiration is slight in most cases, but in a few cases, respiration is quite strongly depressed. For example, 0.01 *M* sulfanilamide has practically no effect against the respiration of *Staph. aureus*, *Strep. hemolyticus*, *E. coli* or *Ps. aeruginosa*. However, Irgafen strongly depresses the respiration of the staphylococci and streptococci, while not affecting the coliform organisms or *Ps. aeruginosa*. With 0.01 *M* sulfacetamide the effect is reversed, there is a marked depression in the respiration of the coliform organisms and *Ps. aeruginosa*, while the staphylococci and streptococci are scarcely affected. When concentrations of the specifically active drugs are less than 0.01 *M*, the effects on respiration are proportionately diminished.

"Sensitivity spectra" are also presented, showing the presence or absence of growth after 48 hours at 37°C., when different concentrations of cells are inoculated into the medium containing 0.01 *M* concentrations of the drugs. Here too, the effects are quite specific, and there is a moderately good correlation with the effects upon respiration. All of the drugs prevent the growth of moderate to heavy inocula of *Staph. aureus*, but only Irgafen and Marfanil have such an action against *Strep. hemolyticus*.

Hirsch considers that his results are of importance in suggesting that when high concentrations of sulfonamides are applied topically to surface injuries, the drugs may show considerable specificity of action. He suggests that combinations of several suitable sulfa drugs should be used, to increase the probability that infective organisms will be exposed to sulfonamides to which they are specifically sensitive. On the other hand, he considers that his results give no proof of any drug specificity with the much lower concentrations obtainable in the blood stream in systemic infections (under which conditions the drugs act against the *p*-aminobenzoic acid mechanisms of the bacterial cells).

The respiration studies may be adversely criticized on the grounds that a depression of bacterial respiration *in vitro* is not necessarily correlated with bacteriostasis *in vivo*. The "sensitivity spectra" are considerably more convincing. However, before any definite conclusions can be drawn as to the specificity of sulfonamides in surface applications, the results of Hirsch need to be checked by animal experiments. If specificity occurs under such conditions, and if mixtures of sulfa drugs show increased efficacy in restraining mixed infections in surface injuries, important improvements in therapy may result.

WALTER C. TOBIE, Stamford, Conn.

**Enzymes and Their Role in Wheat Technology.** Issued by the American Association of Cereal Chemists. Edited by J. A. ANDERSON, Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg. Interscience Publishers Inc., New York, 1946. ix + 371 pp. Price \$4.50.

In the early days of the reviewer's association with cereal chemistry—about thirty years ago—there were few books on the subject. It has been his good fortune to have been active in this branch of science during a period of great advances. Although general books covering the whole subject of cereal chemistry exist, it has been obvious for some time that more specialization and more exact knowledge is wanted. The American Association of Cereal Chemists is a strong and progressive body and cereal chemists the world over will be grateful that the Association have taken the step of issuing a series of monographs. The volume under review is the first of this series and it sets a high standard for the others to follow. The reviewer suggests that nowhere can be found in one compact volume such a useful collection of information on the particular subject.

The scheme adopted in the present monograph has been to divide it into eleven sections. Firstly, there is a general survey of the broad chemistry of enzymes by Sandstrom. Then the various branches of enzymology, such as the amylases, the esterases, the oxidases, the proteases, alcoholic fermentation, are each treated by a contributor who is an authority on the subject. The reader is thus able to appreciate the general state of knowledge on that particular matter. Following each general chapter, there is a chapter on the application of that portion of enzymology to cereals with special reference to milling and baking and again each chapter is written by someone who has specialized in the particular branch. The whole effect is, therefore, that the subject is extremely well covered, although some repetition is unavoidable.

The fact that the monograph is written by no less than fifteen contributors might have resulted in considerable unevenness, but this has to a large extent been prevented by the editorship of Dr. J. A. Anderson, whose qualifications so to act are not far from unique. We know of few people who have the knowledge and the ability to have undertaken that task.

The book is well printed and is remarkably free from errors. There is little to criticize, but, as a cereal chemist, one is sorry that the term "wheat berry" is used in the first chapter and has escaped the eagle eye of the editor.

The monograph teems with references which are, however, not all up to date. Chapter II (on analyses) alone contains about three hundred references. A book

of this kind is naturally heavy going and one could have wished that some sort of summary of the present position of each section might have been attempted for the sake of the general body of cereal chemists, as opposed to those whose work is mainly research.

The real value and excellence of this volume is such that all interested in cereals will be looking forward impatiently to the other volumes of the series and the monograph will certainly add to the prestige of the American Association of Cereal Chemists.

Concluding, as the reviewer commenced, as one who has been studying cereal chemistry longer than most chemists in the milling and baking industries, and who has done his share in writing books on the subject, it has been a privilege to have seen such a book published, and to have had an opportunity to review it. No modern cereal chemist in any part of the world can afford to be without it.

D. W. KENT-JONES, *Ealing, England*

**Embryologie Chimique.** By JEAN BRACHET, University of Brussels. Masson & Co., Paris, 1944. 505 pp. Paper covered. Price 420 francs.

This volume is one of the most important in the field of chemical embryology since the monumental treatise by Needham, published in 1931. The last fifteen years have witnessed a perceptible change in the orientation of scientific thinking in the field of chemical embryology. The development of more precise methods of biochemical analysis, and the refinement of certain tools of physics, have resulted in the transition of chemical embryology from a discipline emphasizing a description of possible relations of composition to morphology, to one in which physical and biochemical alterations are measured to permit a correlation of structure and function during the various stages of embryological development. The topics discussed by Brachet reflect this dynamic viewpoint of chemical embryology.

The first chapter is entitled *Problems and Methods* and is introduced by a short section in which Brachet indicates the nature of the various problems, e.g., types of chemical reactions, energy alterations, and significance of movements of cellular constituents, which are to be considered in relation to determination of sex, formation of gametes, gastrulation, organization, etc. This is followed by a description of available cytochemical and histochemical methods for approaching these problems, with deserved consideration of the histochemical and micromanometric techniques developed by Warburg, Linderström-Lang and his associates, Gerard and Hartline, Fenn, Heatley, Berenblum and Chain, Needham and his colleagues, and other investigators. The chapter contains illustrations of apparatus and, like the other chapters in the book, has an excellent bibliography.

The second chapter is entitled *The Chemical Basis of Sex Determination*. This chapter is devoted largely to a consideration of the results of the collaboration of the botanist F. Moewus, with the biochemist, R. Kuhne, culminating in the demonstration of the nature of the chemical substances responsible for sex determination and attraction of gametes in certain unicellular algae. This chapter has a brief section on the role of the steroid sex hormones in the determination of sex in vertebrates, and closes with a short consideration of possible relations between respiratory metabolism and sex determination.



Chapter three is entitled *The Formation of Gametes*, and after a preliminary description of staining reactions seen during egg development, presents a careful description of the author's investigations, and those of other workers, notably Caspersson and his colleagues, on variations in nucleic acid concentration and distribution and their possible significance. The next section of this chapter includes the detailed biochemical information which has been obtained in studies of the egg, and the chapter closes with a consideration of biochemical modifications observed during spermatogenesis.

The fourth chapter is a lengthy presentation of *The Biochemistry of Fertilization*. The author discusses the role of the echinochromes in determining the chemotaxis of spermatocytes. The substances called gynogamones and androgamones are also mentioned, as is the possible role of glutathione in fertilization. This is followed by a detailed consideration of the alterations seen in the metabolism of the egg as a consequence of fertilization, with carbohydrate and protein metabolism (including products derived from proteins) receiving attention in special sections of the chapter. The author concludes the chapter with a consideration of the various theories of fertilization in relation to the biochemical data which have been discussed.

The title of chapter five is *Segmentation, Metabolism and Cellular Division*. The first portion of this chapter deals largely with alterations in respiratory metabolism seen at various stages of cellular division. This is followed by a consideration of non-respiratory metabolic features of cells during mitosis. Considerable attention is given to the role of proteins, and to certain configurations in the protein molecule, particularly the sulfhydryl group, in the morphological changes seen in the cell during division. The emphasis here is perhaps greater than is justified by the existing experimental data.

The author then returns to the nucleic acids in chapter six which is entitled: *Synthesis, Localization and Physiological Role of the Nucleic Acids*. This is a comprehensive survey of many investigations, including those of the author, which have yielded data of significance for future work in fundamental cell biochemistry. Attention is given particularly to the work and concepts of Caspersson and his colleagues, with a critical evaluation of the limitations of the techniques and therefore of the data upon which certain hypotheses have been based. As the author indicates, it is apparent that the significance assigned to studies of the nucleic acids in cellular development exceeds that justified by the limitations of the experimental approach. While it may be expected that an important constituent of cell nuclei and cytoplasm should play a prominent role in cellular growth and differentiation, unequivocal proof for this role, and its nature, remain to be obtained in future investigations.

Chapter seven is called *Growth, Differentiation and Metabolism* and deals largely with a discussion of the meaning of the data of respiratory metabolism in terms of possible sources of the energy which is required for the various stages of cellular development. Chapter eight is a lengthy consideration of the *Chemical Embryology of Invertebrates*, and chapter nine is entitled *Chemical Embryology of the Brachian Egg*. The next two chapters in the book are headed *The Metabolism of the Organization Center* and *The Chemical Nature of the Inductor Substance, and Organization, Induction and Biochemistry*, with a final chapter called *Acquired Facts, Controversial Points and Future Prospects*. These last three chapters contain much stimulating material

presented by the author in a style that includes provocative questions. The book has a subject index and a valuable bibliography of over one thousand references.

The reading of this book has been a stimulating experience. Certain fundamental problems posed by the author are, when viewed against the background of modern biochemistry, susceptible to experimental study. The next decade of investigations in chemical embryology will undoubtedly prove a fruitful one, and the basic nature of the processes concerned should increase the value of these contributions in many aspects of biological research. The author is to be congratulated on producing, during a period of war privations, a splendid and useful discussion and summary of the existing recent knowledge and the important unsolved problems of chemical embryology.

ABRAHAM WHITE, New Haven, Conn.

**Physikalische Chemie in Medizin und Biologie.** W. Bladergroen, Wepf & Cie. Publishing Company, Basel, Switzerland, 1945. 476 pages.

The author attempts in this book to present a review of all branches of fundamental physical chemistry applicable to the interpretation of biology and medicine, and of all the applications which actually have been made to account for biological processes. This very exacting goal has been achieved most successfully. The fundamental physical chemistry has been represented in an entirely up-to-date manner, the biological and medical part of the book is of vast circumference. The book is not to be considered as a comprehensive text book but rather as a general review of our present-day knowledge. It will serve as an inspiring introduction for biologists as well as clinicians into the realm of physico-chemistry. This latter concept is not taken in a too strict sense. Many chapters, especially those on metabolism, comprise numerous aspects which can be just as well allocated to the field of biochemistry, or better of that branch of organic chemistry which the living cell has decided to utilize for its purpose, and which, as regards the methods of synthesis and breakdown processes, are often so very different from what text books of classic organic chemistry have taught the student. The vast multitude of topics discussed makes it practically impossible to give a critical review of the contents. It may suffice to enumerate the various headings of the chapters: Atoms, ions and molecules; Thermodynamic considerations; Aqueous solutions; Acid-base equilibria; Interface phenomena; Disperse systems; The structure of the living substance; Colloid-chemical processes in the organism; Important osmotic processes; Oxidation-reduction potentials; Problems of Metabolism; Biological oxidations. I am sure that individual experts in any one of the fields discussed may present the subject matter in a different way, considering the rapid evolution of science, which makes almost any review almost obsolete when perused a year after its writing, not to speak of the date of publication. Every biologist and every clinician will enjoy studying this book.

L. MICHAELIS, New York, N. Y.

**Zur Chemie, Physiologie und Pathologie des Eiweisses.** By R. SIGNER, H. THEORELL, I. ABELIN AND E. GLANZMANN. Verlag Paul Haupt, Bern, 1944. 155 pp.

This is a group of four lectures delivered at the *Berner Biochemisches Vereinigung* in the summer of 1943 and published with a brief introduction as a reprint from the

*Mitteilungen der Naturforschenden Gesellschaft Bern.* The first, on the chemistry and colloid chemistry of the proteins, by Signer, seems to have been hastily put together. The material for discussion is far from well chosen, there are many misleading if not inaccurate statements and there is no clear development of the central theme. Only brief and inadequate discussions of a series of disconnected topics are given.

By contrast, Theorell's lecture on the constitution and activity of hemin proteins is a clear and well-documented review of this important field. He takes up hemoglobin and myoglobin, the cytochromes, catalases and peroxidases, deals with their points of similarity and dissimilarity and, so far as is possible, develops a theoretical treatment of their behavior in terms of the structure of the iron-containing group. He clearly points out the limitations of our present knowledge and the directions in which future research is required.

Abelin's lecture on the physiology of protein metabolism is likewise a review but from a point of view that places great emphasis upon the specific structure of tissue proteins. Thus he takes up the speculations of Alcock, of Larmour and of Block, although with full realization of their highly preliminary nature, and points out their bearing upon the facts regarding essential amino acids. Specific dynamic action is discussed in terms of the parallel between the effect of proteins in nutrition and of the action of certain drugs such as adrenalin. More than a quarter of the space is devoted to the so-called reserve-protein of the body.

The last lecture, by Glanzmann on the pathology of protein metabolism, reviews the hypoproteinemias, the hyperproteinemias and the dysproteinemias, or toxicities of protein origin, in a clear and well-organized manner. The material is dealt with from the clinical point of view, emphasis being placed upon the importance of the disturbances of metabolism in which proteins can be shown to play a part.

The book as a whole, with the exception of the first lecture, can well serve as assigned reading in courses in biochemistry where additional drill in German is desired. It is not an outstanding contribution but it may well prove to be a useful one.

H. B. VICKERY, *New Haven, Conn.*

**Micro-Analysis in Medical Biochemistry.** By E. J. KING, Professor of Chemical Pathology in the University of London at the British Post-graduate Medical School. J. and A. Churchill, Ltd., London, England, 1946. viii + 168 pp. Price 10 s. 6 d.

This concisely written book describes procedures for the quantitative estimation of the components of blood, urine, spinal fluid, feces, gastric contents and calculi, most frequently requested by the clinician. One chapter is devoted to each of the following topics: tests of function, measurement of pH, the preparation of volumetric solutions and colorimetry.

The author embodies a number of micro methods emanating from his laboratory for the analysis of a given constituent on 0.2 ml. of blood. He accomplishes this in most cases by the reduction of the final volume of colored solution which is read in the visual colorimeter with the aid of light filters and microcups. For titrimetric methods the usual 5 or 10 ml. microburet is employed. In some cases the amount of blood recommended by the original author of the method is employed (*i.e.*, 2 ml. for Ca, 1.0 ml. for CO<sub>2</sub>). The use of the photoelectric colorimeter is discussed but no advantage of it is taken in presenting methods for still smaller volumes of blood

which are used in some of the laboratories. The description and use of the capillary microburet is completely omitted.

For single determinations the author describes methods which employ fingertip blood. There are advantages in this for the patient if used with discrimination. The clinical significance of some determinations (which are described for fingertip blood) like cholesterol and inorganic phosphorus, is masked by whole blood analysis instead of serum or plasma. Barring such limitations, the use of fingertip blood is a desirable practice for single determinations and using "Ultramicro methods" where only 0.004 to 0.100 ml. of blood is required it can be used for multiple determinations. This new development was possible by the use of the spectrophotometer for colorimetric methods and the capillary microburet for titrimetric procedures. The author omits these "Ultramicro Methods" which in view of recent trends would be expected in a new book. However, in all fairness to the author, the use of 0.2 ml. of blood is less than that employed by most of the clinical laboratories today.

The methods are all presented in a descriptive manner. The silent assumption is made that the user of the methods is not interested in the fundamental theory of the instruments and apparatus nor the chemical reaction and physical principles underlying the methods. In this respect the book is similar to most of the books published on the subject of micro analysis in the clinical laboratory. This is an unfortunate tendency since there is a distinct need for a book on the subject that would discuss the fundamental principles of micro methods, as this field is no longer the domain of the technician or the person with training mostly in the biological sciences, but for persons well trained in chemistry and physics as well.

ALBERT E. SOBEL, *Brooklyn, N. Y.*

**Vitamines et carences alimentaires.** Par GEORGES MOURIQUAND, Professeur à la Faculté de Médecine de Lyon. Pp. 462, with 65 illustrations. Albin Michel, Paris, 1942.

This book is divided into two major portions. In the first division, the author presents a discussion of vitamin A, thiamine, ascorbic acid, vitamin D, and nicotinic acid. In addition to this, short chapters on riboflavin, pyridoxin, and vitamins H, E and K, have been included. The second part of the book is entitled "The great nutritional problems of today" and consists of three divisions. The first deals with the nutritive equilibrium; the second discusses the nutritive requirements of the body, and the third gives tables showing the vitamin contents of foods.

The title of this book may lead the reader to expect a treatise covering the whole field of vitamins. Instead the author has confined his discussion, at least in the first part of the book, to his own investigations. The main fields of research of Mouriquand and his associates may be seen from the length of the various chapters. The topics covered are as follows: vitamin A (42 pp.); vitamin C (74 pp.); vitamin D (52 pp.); thiamine (54 pp.); nicotinic acid (28 pp.); vitamins E and K (4 pp. each); riboflavin (2 pp.); pyridoxin (less than one page); vitamin H (biotin) (less than one page).

This book was written in 1941. The most recent survey of the vitamin field, published outside of France, to which reference is made in the text, appeared in 1938. No mention is made of choline, or of pantothenic acid, and biotin is still called vitamin H. Very little space is devoted to the chemical and physical properties of

vitamins. In spite of these shortcomings, the book contains a wealth of material based on more than 25 years of research by one of France's foremost workers in the field of vitamins. Since the author is a pediatrician, emphasis has been placed in the first part of the book on the symptomatology caused by the various avitaminoses in children. However, the experimental approach has not been neglected. In this connection, the following statement made by Mouriquand in one of his articles (*Presse Médicale* 42, 371 (1934)) might have served as motto to the book "Nous n'avons cessé de nous inspirer de la clinique pour provoquer l'expérimentation et de l'expérimentation pour éclairer la clinique." Thus, the book should appeal specially to two types of readers, to the clinician and to the experimentalist. The clinician will be interested (a) in Mouriquand's findings with regard to the antagonism between thyroxine and vitamin A, thiamine, and vitamin C, respectively; (b) his observations dealing with the greater susceptibility to injury from injections of diphtheria toxin in guinea pigs deficient in vitamin C; (c) his studies on the accelerated dentition produced in children by excessive doses of vitamin D; (d) his studies of the developmental stages of scurvy and rickets leading to a distinction between eutrophic and dystrophic phases of these diseases; (e) his observations on the greater susceptibility of artificially fed infants to rickets as compared with breast-fed infants; (f) his discussion on dietary poisons (diétotoxiques), substances which have no harmful effects when the composition of the diet is normal, but which act as poisons as soon as some constituent of the diet is omitted.

The experimentalist will probably find his main interest in (a) Mouriquand's experiments on the effect of ultraviolet light on the cornea of vitamin A deficient rats as compared with its effect on normal rats; (b) his observation that injection of diphtheria toxin in guinea pigs causes a drop in the ascorbic acid and sterol content of the adrenals; (c) his demonstration that in the second half of pregnancy the guinea pig is immune to scurvy; (d) his studies on the hypertrophy of the spleen produced in rats suffering from rickets; (e) his claim that cod liver oil interferes with the cure of scurvy in guinea pigs; (f) his experiments showing that the water intake influences the development of scorbutic symptoms in the guinea pig; (g) his success in producing dystrophic rickets in rats by adding to a rachitogenic diet either strontium carbonate or magnesium carbonate.

On the whole, the author has made an important contribution. It is to be hoped, however, that in a future edition it will be possible to enlarge its scope so as to include for discussion the work carried out in other countries. Also, a detailed index would add to the usefulness of the book.

L. R. CERECEDO, *New York, N. Y.*

# Amino Acid Fermentations by *Clostridium propionicum* and *Diplococcus glycinophilus*

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## INTRODUCTION

Several anaerobic bacteria are known to satisfy their energy requirements by decomposing amino acids. Two types of catabolic reactions have been reported. Some organisms, like *Clostridium sporogenes* (1) and *Cl. botulinum* (2), cause oxidation-reduction reactions between pairs of different amino acids. For example, alanine or leucine may be oxidized and glycine or proline reduced. Other anaerobic bacteria, like *Cl. tetanomorphum* (3, 4) and *Cl. tetani* (5, 6), cause fermentations of single amino acids such as glutamic and aspartic acids, serine, threonine, cysteine and histidine. Until recently none of the simple unsubstituted amino acids has been found to be readily fermented. It has even been stated that a fermentative breakdown of simple amino acids would be impossible in the absence of an outside oxidizing or reducing agent (1). In spite of this opinion we were able to isolate a glycine-fermenting coccus, *Diplococcus glycinophilus*, and an alanine-fermenting bacterium, *Clostridium propionicum*, from black mud by the enrichment culture method (7, 8). The present paper deals with the decomposition of amino acids and related non-nitrogenous compounds by these two bacteria.

## CLOSTRIDIUM PROPIONICUM

### *Methods*

Unless otherwise stated the experiments were done with washed cell suspensions. The bacteria were grown anaerobically in the following medium: alanine 0.3 g., Bacto peptone 0.3 g., Bacto yeast extract 0.4 g., M/1 phosphate buffer, pH7, 10.5 ml., saturated solution of calcium sulfate 0.25 ml., cysteine hydrochloride (neu-

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tralized) 0.05 g.,  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  0.005 g.,  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$  0.001 g., glass-distilled water 100 ml. Cultures were harvested after 16 hours at 37°C. or 24 hrs. at 30°C. The bacteria were separated by centrifugation and washed twice with *M*/20 phosphate buffer pH 7.2 containing 0.02%  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ . The cells from 1 liter were finally suspended in 15 ml. of phosphate buffer. Such a suspension normally contains 1.0 to 1.2 mg. of cell nitrogen/ml. The initial  $Q^{(N)}_{\text{alanine}}$  is about 2,000. The cells remain active for 3-4 days when stored anaerobically at 5°C.

The pH range of activity of cell suspensions in decomposing alanine is from about 5.5 to 9.0. The pH-rate curve is almost symmetrical with an optimum between 7.1 and 7.4.

### *Compounds Decomposed*

Using the Warburg manometric technique, the fermentability of a number of compounds was tested in a nitrogen atmosphere at 37°C. Unless otherwise stated, 0.2 ml. of a *M*/10 solution of the substrate was added to 1.8 ml. of a cell suspension. The formation of gas was taken as an indication of fermentability. With nitrogenous compounds, ammonia was also determined at the end of the experiment. Compounds not attacked alone were tested again in the presence of an equimolar quantity of alanine.

The compounds fermented readily are *dl*-alanine, *dl*-serine, *dl*-threonine, pyruvate, acrylate, *l*-cysteine and *dl*-lactate. *dl*-Methionine is decomposed more slowly. *dl*-Valine and *l*-aspartate are not attacked alone but are slowly broken down to give carbon dioxide and ammonia when supplied together with alanine.  $\alpha$ -Aminobutyric acid yields the same products at a barely detectable rate even in the presence of alanine. Compounds not decomposed at a significant rate either in the presence or absence of alanine are *l*-tryptophane,  $\beta$ -alanine, *dl*-glutamate, *dl*-phenyl alanine, glycine, *l*-hydroxyproline, *l*-proline, *dl*-lysine, *dl*-leucine, *l*-tyrosine, *dl*-arginine, *l*-histidine, glycerol, glycerate, *dl*-malate, dihydroxyacetone, glyceric aldehyde, fumarate, glycolate and glucose.

The relative rates of decomposition of several of the more readily utilizable substrates are illustrated in Fig. 1. It can be seen that acrylate is decomposed much more rapidly than the other compounds and alanine is attacked next most rapidly. Lactate, pyruvate and serine are all metabolized at about the same rate but more slowly than alanine. The rates of carbon dioxide evolution from acrylate, alanine and lactate remain almost constant until the substrates are completely used up. With serine and pyruvate, however, the rates slow down gradually as the substrate concentrations decrease.

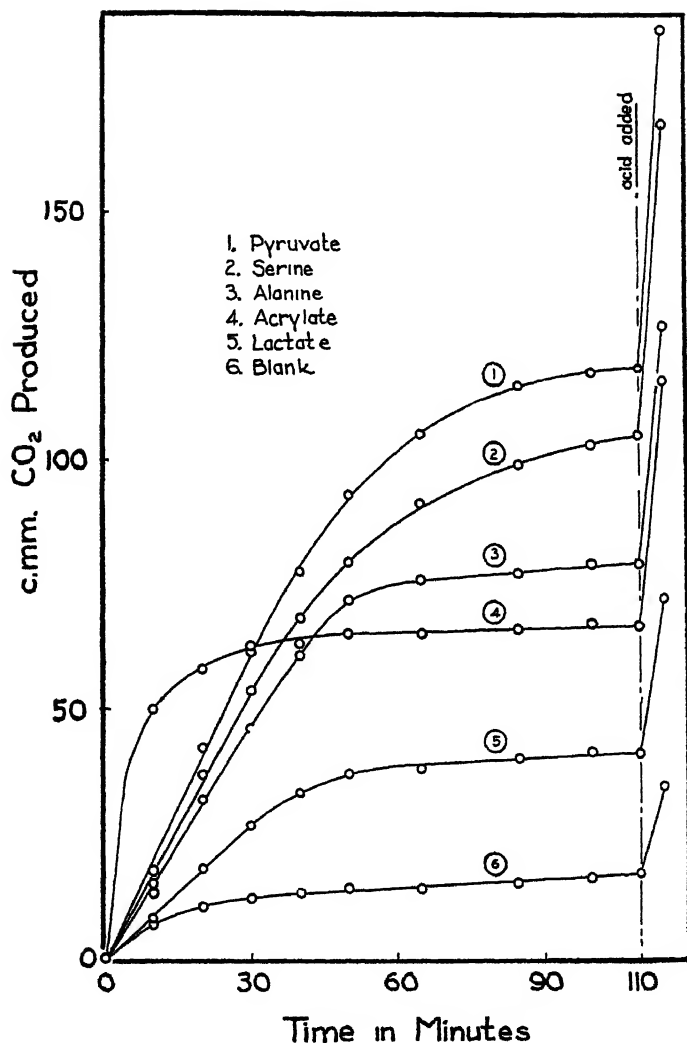


FIG. 1. Relative Rates of Decomposition of Various Substrates by *Clostridium propionicum*. 37°C. 1.8 ml. cell suspension + 0.2 ml. *M*/10 substrate (except lactate, 0.1 ml. *M*/10). In comparing the rates of decomposition it should be noted that 2 moles of carbon dioxide are formed per mole of pyruvate and serine, while only 1 mole of carbon dioxide is formed per mole of the other substrates.



*Fermentation Products*

The products of the fermentation of alanine were first identified and determined quantitatively using a growing culture. The medium was the same as that given above except that the only organic constituents were 0.65% alanine and 3 vol.-% yeast autolyzate. The cysteine was replaced by 0.03% sodium sulfide. The culture was incubated 7 days at 30°C.

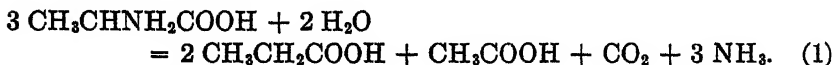
Ammonia production was used as a measure of alanine decomposition since preliminary experiments had shown that very little ammonia is formed from yeast autolyzate alone and the increase in ammonia is equivalent to the decrease in amino nitrogen. Acetic acid was identified microchemically as sodium uranyl acetate. Propionic acid was identified as the silver salt. The presence or absence of butyric acid was proved by the azeotropic distillation method of Schickel *et al.* (9). Quantitative determination of volatile acids was done by Duclaux distillation. No neutral volatile compounds or non-volatile acids could be detected.

TABLE I

*Fermentation of Alanine by a Growing Culture of Cl. propionicum*  
(mM/100 mM alanine decomposed)

Product	mM
Ammonia	(100)
Carbon dioxide	32.1
Acetic acid	30.8
Propionic acid	61.5
Carbon recovery ( <i>per cent</i> )	93
Redox index	0.96

The results of a typical analysis are given in Table I. The data show that *Cl. propionicum* causes a propionic acid type fermentation of alanine which corresponds to the equation



The fermentation products of serine, threonine, alanine, pyruvate, lactate and acrylate were determined by the use of cell suspensions. The technique was essentially as follows: A suitable quantity of substrate, usually 100–200 mg., was weighed into a Thunberg tube and 10 ml. of a cell suspension were added. In the side arm was placed 0.1 ml. of saturated carbon dioxide-free sodium hydroxide solution. The tube was then rapidly evacuated and incubated at 37°C. for six hours. The alkali was then mixed with the cell suspension to fix all gaseous carbon dioxide after which the contents of the tube were diluted to a convenient volume and analyzed. A blank treated in the same manner, except for the absence of substrate, was analyzed at the same time and suitable corrections were made.

Table II gives the results of these experiments. The carbon recoveries and oxidation-reduction balances are satisfactory except for acrylate which gave only an 89% carbon recovery. The explanation of this result is uncertain. The acrylic acid was purified by steam distillation. It reacted with the theoretical quantity of bromine and was very rapidly and apparently completely fermented. However, there is a pos-

TABLE II

*Fermentation of Various Substrates by Cell Suspensions of Cl. propionicum*  
(mM of products/100 mM of substrate decomposed)

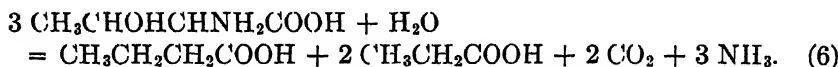
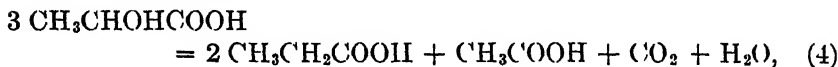
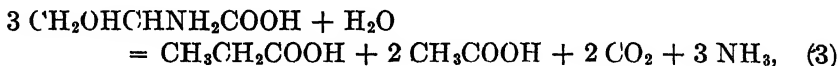
	Alanine	Pyruvate	Lactate	Serine	Acrylate	Threonine
Ammonia	100	—	—	100	—	100
Carbon dioxide	34.0	61.6	36.5	65.9	30.6	74.8
Acetic acid	32.6	66.7	33.0	66.2	29.1	—
Propionic acid	66.4	33.3	66.0	33.1	58.2	67.4
Butyric acid	—	—	—	—	—	33.7
Per cent C recovered	100	98	100	99	89	102
Redox index	1.02	0.92	1.10	0.99	1.05	1.11

sibility that some of the acrylate may have polymerized to non-fermentable, non-volatile products. Except for the low carbon recovery, the data are in good agreement with equation 5 below since the ratio of carbon dioxide to volatile acid is 0.35 and the ratio of propionic to acetic acid is 2.0.

In a preliminary report on this work (7), it was stated that the volatile acids formed from threonine are butyric and acetic. This conclusion was reached on the basis of Duclaux distillation data only.<sup>1</sup> Subsequently, the volatile acids were reexamined by a combination of the azeotropic and Duclaux distillation methods and it was found that the second acid is propionic and not acetic. In several experiments the ratio of butyric to propionic acid varied from 1:1.8 to 1:2.2, the average being 1:2.0.

<sup>1</sup> From such data obtained with mixed volatile acids it is not possible to decide whether butyric and propionic acids are present in a ratio of 1:2 or whether butyric and acetic acids are present in a ratio of 2:1 as was assumed in the earlier report. Neither the percentage carbon recovery nor the redox index is appreciably different for these two fatty acid mixtures. This stresses the importance of using care in interpreting Duclaux distillation data.

The following equations fit the analytical data of Table II rather closely:



### *Hydrogen Donors to Methylene Blue*

The ability of several amino acids to serve as hydrogen donors was tested by the usual "Thunberg" technique (10). In a typical experiment the following decolorization times (in minutes) were observed at 37°C.: *l*-glutamic acid 1.5, *dl*-alanine 2.0, *dl*-valine 3.5, *dl*-leucine 3.75, *l*-phenyl alanine 6.5, *l*-cysteine 7.0, glycine 10, *l*-histidine 25, control (no substrate) 60, *l*-proline 60, and *l*-aspartic acid 60. It is evident that *Cl. propionicum* possesses dehydrogenases for several amino acids that are not rapidly broken down to yield carbon dioxide or ammonia under the conditions of the previous experiments.

### DISCUSSION

The experimental results show that *Cl. propionicum* decomposes alanine, lactate, acrylate, serine and pyruvate by a propionic acid type fermentation. Part of each substrate is oxidized to acetic acid and carbon dioxide while another part is reduced to propionic acid. The relative amounts of the two volatile acids depend, of course, on the state of oxidation of the substrate. Alanine, lactate and acrylate, which are all in the same state of oxidation, yield propionic and acetic acids in a ratio of 1:2. The decompositions of nitrogenous and non-nitrogenous substrates of the same oxidation state appear to differ mainly with respect to the formation of ammonia. In this connection

it is of interest to note that the true propionic acid bacteria of the genus *Propionibacterium* are unable to catabolize either amino acids or acrylic acid (11). *Cl. propionicum* also differs from the propionic acid bacteria, but resembles other obligately anaerobic bacteria, with respect to its inability to form succinic acid.

The mechanism of formation of propionate from lactate and alanine is not known (11). It has been suggested that the first step in this conversion could be a dehydration giving acrylate. This explanation is not tenable in the case of fermentations by species of *Propionibacterium* since these organisms are unable to utilize acrylate. With *Cl. propionicum*, on the contrary, the very rapid conversion of acrylate to propionate gives some support to this idea. However, at present there is no direct evidence for the formation of acrylate from either lactate, alanine or pyruvate.

The formation of butyric acid from threonine is of special interest. All the other substrates studied give propionic and acetic acids, while threonine gives propionic and butyric acids. It is very unusual for a bacterium to produce different fatty acids from different fermentable substrates. The explanation must be that the butyric acid is not formed by a condensation of two molecules of acetic acid, the usual mechanism (12, 13), but by a direct reduction of the threonine molecule. If a condensation of acetic acid were involved, one would expect butyric acid to be formed also from  $C_3$  substrates. This does not occur.

Propionic acid must be formed from threonine by oxidation in the same way that acetic acid is formed from  $C_3$  substrates.

## DIPLOCOCCUS GLYCINOPHILUS

### Methods

The preparation of cell suspensions was essentially the same as for *Cl. propionicum*. The bacteria were grown anaerobically in the same medium, except that the organic constituents were glycine 0.3%, Bacto peptone 0.5% and Bacto yeast extract 0.5%. Cultures were harvested after 48–72 hours at 30°C. The cells are very stable, retaining almost their full activity *in vacuo* at 5°C. for 10–14 days. For manometric experiments it was found desirable to use a quantity of cells containing at least 0.4 mg. N with 0.2 ml.  $M/10$  glycine in a total volume of 2–3 ml. Under these conditions the  $Q(N)_{\text{glycine}}$  is 1,000–1,300 at 37°C. With more dilute suspensions the rate is much lower and the decomposition is sometimes incomplete.

The optimum pH for glycine decomposition by cell suspensions is about 7.2, the pH range being from 5.5 to 9.0.

### Compounds Decomposed

The fermentability of a variety of amino acids and non-nitrogenous compounds was tested by the same method used with *Cl. propionicum*. The substrates were usually added simultaneously with an equimolar quantity of glycine. Decomposition of the test substance was indicated by gas or ammonia production in excess of the amount formed from glycine alone. The following compounds were tested: glycine, *dl*-alanine, *dl*-serine, *dl*-valine, *l*-proline, *l*-hydroxyproline, *l*-tyrosine, *dl*-lysine, *dl*-isoleucine, *l*-histidine, *dl*-norleucine, *l*-cysteine, *dl*-methionine, *l*-tryptophan, *l*-threonine, *dl*-arginine, *dl*-phenylalanine, *dl*-aspartate, *dl*-glutamate, *l*-asparagine, *p*-hydroxyphenylglycine, *d*-glucosamine, urea, thiourea, glucose, pyruvate, *dl*-lactate, glycerate, acrylate, glycerophosphate, glycolate, glyoxylate, malate, formate, succinate and fumarate.

The only compound decomposed rapidly by *D. glycinophilus* under the experimental conditions is glycine. Serine and pyruvate are also attacked in the presence of glycine, but much more slowly. About 10% of the serine nitrogen is liberated as ammonia in the time required to decompose completely an equimolar amount of glycine. The addition of serine caused no appreciable increase in gas production. With pyruvate 10–15% more gas is formed than with glycine alone. Neither serine nor pyruvate is decomposed in the absence of glycine.

### Products of Glycine Fermentation

To determine the products of glycine decomposition by growing cultures a medium containing 0.5% glycine, 3 vol.-% yeast autolyzate and the usual inorganic salts was used. The culture was incubated anaerobically for 7 days at 30°C.

The analytical results are given in Table III, column 2. The quantity of glycine decomposed is calculated from the ammonia production. Approximately 3 moles of acetic acid and 2 moles of carbon dioxide are produced for each 4 moles of glycine decomposed. Therefore the equation for the reaction is



This equation correctly represents the decomposition of glycine only in stationary cultures where the gas space in contact with the medium is relatively small.

TABLE III  
*Fermentation of Glycine by D. glycinophilus*  
 (mM/100 mM glycine decomposed)

Product	Stationary growing culture	Cell suspension on shaker	Cell suspension in respirometer vessel
Ammonia	(100)	(100)	(100)
Hydrogen	none observed	not determined	38
Carbon dioxide	50.2	93.0	70.2
Acetic acid	72.0	52.6	62.0
Carbon recovery (per cent)	97	99	96
Redox index	1.01	1.85	1.02

The vessel used for the above experiment was a 100 ml. volumetric flask filled to the mark. When a fermentation is carried out in a vessel with a large gas space, particularly when the medium is shaken continuously, quite different results are obtained. For example, in one experiment 10 ml. of a cell suspension of *D. glycinophilus* was shaken with 200 mg. of glycine in an evacuated 300 ml. flask until the substrate was completely decomposed. The reaction mixture was then analyzed for ammonia, acetic acid and carbon dioxide with the results shown in column 3, Table III.

Under these conditions the yield of carbon dioxide is almost doubled and the yield of acetic acid is markedly decreased as compared to a stationary growing culture. Although acetic acid and carbon dioxide account for all the carbon in the glycine decomposed, the redox index is very high, suggesting that hydrogen is formed as an additional product.

The formation of an alkali-insoluble gas from glycine was demonstrated in a manometric experiment using a nitrogen atmosphere. The gas was identified as hydrogen by showing that it could be quantitatively absorbed by 100 mg. of palladinized asbestos treated with 0.1 ml. of a saturated aqueous solution of methylene blue. The palladinized asbestos reagent was placed in the center well of the Warburg vessel. Under these conditions the absorption of hydrogen is slow, 6-8 hours being required to completely absorb 250 mm.<sup>3</sup> at 37°C.

The results of an experiment to determine quantitatively the products of glycine fermentation in Warburg vessels with a nitrogen atmosphere are given in column 4, Table III. Rather a large amount of hydrogen gas (38 mM/mM glycine) was formed. The hydrogen gas

and other products account for all the hydrogen, carbon and nitrogen of the substrate.

*Influence of  $pH_2$  on Hydrogen Formation from Glycine*

It is evident that the yield of hydrogen gas and therefore the yields of acetic acid and carbon dioxide as well depend on the experimental conditions, more particularly on the ratio of liquid to gas volumes and the extent to which the culture is shaken. This suggested that the essential factor controlling the formation of hydrogen is its partial pressure. This possibility was first explored by comparing the yields of hydrogen in manometer vessels in which the gas space was filled with nitrogen and hydrogen, respectively. The results were quite conclusive. In the nitrogen-filled vessel there was a considerable formation of hydrogen while in the hydrogen-filled vessel no hydrogen was evolved. In some, but not all, experiments, a small amount of hydrogen was actually absorbed from a hydrogen atmosphere.

Having established the fact that hydrogen production is prevented completely by a hydrogen atmosphere, it next seemed desirable to study the influence of different hydrogen partial pressures. This was done by using various mixtures of hydrogen and nitrogen as the gas phase in manometric vessels.<sup>2</sup> Both hydrogen and carbon dioxide were determined by using two vessels, one with and the other without alkali in the center well.

The results of this experiment are given in Table IV. There was a rapid drop in the amount of hydrogen and carbon dioxide produced from a given amount of glycine as the amount of hydrogen in the gas phase was increased from zero to 25%. At higher hydrogen partial pressures there was a small hydrogen uptake which was not dependent on glycine decomposition since it occurred also in the control without glycine. Half maximal inhibition of hydrogen formation occurs when about 10% hydrogen is present in the gas phase.

The results explain the absence of visible hydrogen evolution in stationary growing cultures. At the beginning of the fermentation, hydrogen is undoubtedly formed. But as soon as the partial pressure of hydrogen in the liquid reaches 25–30% of an atmosphere, *i.e.*, before the pressure becomes high enough to form gas bubbles, hydrogen evolu-

<sup>2</sup> We are indebted to Professor T. F. Buehrer of the University of Arizona for the gas flow meters used in this experiment.

tion stops. Due to the low solubility of hydrogen only a very small quantity need be produced in order to reach this condition. When the ratio of gas to liquid volumes is small, the total yield of hydrogen is negligible.

TABLE IV  
*Hydrogen and Carbon Dioxide Formation from Glycine in*  
*H<sub>2</sub>-N<sub>2</sub> Mixtures by *D. glycinophilus**  
(0.021 mM glycine. Total volume 2.2 ml. T. = 37°C.  
Fermentation time, 100 min.)

H <sub>2</sub> in atmosphere Per cent	H <sub>2</sub> production mm. <sup>3</sup>	CO <sub>2</sub> production mm. <sup>3</sup>
0	201	389
6	122	308
11	63.2	295
23	15.9	248
34	-38.7	268
77	-60.5	241
100	-48.8	225
100 (no glycine)	-52.0	38

In the shaker experiment (Table III, column 3) the gas-liquid ratio was high (35) and the shaking vigorous. These conditions favored the transfer of hydrogen from the medium to the gas phase. A relatively large amount of hydrogen could therefore be formed before the limiting hydrogen pressure was reached.

#### *Utilization of Peptides and Related Compounds*

Several dipeptides, tripeptides and related compounds containing glycine were tested as substrates for *D. glycinophilus* using the manometric technique. The results are given in Table V.

The data show that dipeptides containing glycine with a free carboxyl group, such as diglycine and leucylglycine, are readily decomposed whereas dipeptides containing glycine with a free amino group, such as glycylleucine, and tripeptides, such as triglycine and alanyldiglycine, are attacked very slowly if at all. Hippurylglycine is the only compound containing two peptide linkages that is decomposed fairly rapidly.

Hippuric acid, *p*-aminohippuric acid and glycine yield gas and ammonia at the same rate, indicating that the breakdown of glycine is probably the rate-limiting step. With all three compounds the rate of



gas production is constant until the substrate is completely decomposed. The other dipeptides, *dl*-leucylglycine, diglycine and *dl*-alanylglycine, are attacked more slowly than glycine (Table V, column 2) and the rates do not remain constant but decrease markedly as the

TABLE V  
*Decomposition of Peptides\* by D. glycinophilus*  
(0.02 mM substrate, 37°C, N<sub>2</sub> atmosphere)

Substrate	Relative initial rate of gas evolution	Percentage of total N liberated as NH <sub>3</sub> in 5 hours	H <sub>2</sub> /CO <sub>2</sub>	CO <sub>2</sub> /NH <sub>3</sub>
Glycine	100	100	0.65	0.77
Hippuric acid	100	100	0.66	0.74
<i>p</i> -Aminohippuric acid	100	50	0.69	0.74
<i>dl</i> -Leucylglycine	75	48	0.63	0.68
Diglycine	55	75	0.59	0.71
<i>dl</i> -Alanylglycine	34	45	0.60	0.71
Acetyl glycine	24	65	0.63	0.76
Hippurylglycine	24	80	—	—
<i>dl</i> -Alanyldiglycine	5	<6	—	—
Triglycine	4	<6	—	—
Glycyl- <i>l</i> -leucine	<3	0	—	—
<i>dl</i> -Leucyldiglycine	<3	<6	—	—
Blank (no substrate)	2	—	—	—

\* The authors wish to thank Professor M. J. Johnson of the University of Wisconsin for the gift of several dipeptides.

substrate concentration falls (Fig. 2). This probably accounts for the fact that the theoretical yield of ammonia, one mole for each glycine residue, is not always reached even after long incubation. Leucylglycine and alanylglycine give almost theoretical yields of ammonia, both the *d*- and *l*-isomers being attacked. Diglycine, acetyl glycine and hippurylglycine, on the contrary, are always decomposed incompletely (65–80%) under the experimental conditions used.

To find out whether only the glycine portion of the dipeptides is fermented, the quantity of nitrogen liberated as ammonia and the H<sub>2</sub>/CO<sub>2</sub> and CO<sub>2</sub>/NH<sub>3</sub> ratios were determined (Table V, columns 3, 4, 5). The data show that only glycine is fermented. The quantity of ammonia is never greater than that corresponding to the glycine resi-

due, and the product ratios are in all cases essentially the same as are obtained with glycine itself.

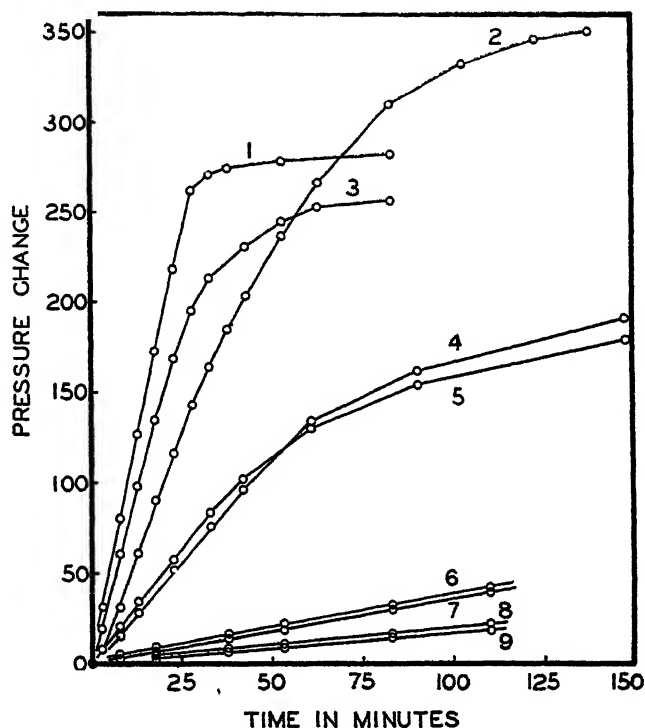


FIG. 2. Relative Rates of Decomposition of Glycine, Di- and Tripeptides by *Diplococcus glycinophilus*. 37°C. 1.8 ml. cell suspension + 0.2 ml.  $M/10$  substrate.  $N_2$  atmosphere. Pressure change is expressed in m.m. Brodie's solution. 1, glycine; 2, diglycine; 3, *dl*-leucylglycine; 4, hippurylglycine; 5, *dl*-alanylglycine; 6, triglycine; 7, *dl*-alanyldiglycine; 8, glycylleucine or *dl*-leucyldiglycine; 9, no substrate.

### DISCUSSION

The most notable characteristic of *D. glycinophilus* is its extraordinary substrate specificity. Most microorganisms are able to utilize at least a few closely related compounds as sources of energy. But this organism appears to be unable to catabolize any compound other than glycine and substances which can be hydrolyzed to glycine. Its specificity in this respect is comparable to that of some of the autotrophic

bacteria. The dipeptidase system also appears to be unique (14) since it acts rapidly only on dipeptides containing glycine with a free carboxyl group.

Previous studies on the catabolism of anaerobic bacteria have shown that glycine can be used in two ways. *Cl. sporogenes* (1) and *Cl. botulinum* (2) use glycine as a hydrogen acceptor, converting it to acetic acid and ammonia. *Cl. acidurici* and *Cl. cylindrosporum*, on the contrary, activate glycine as a hydrogen donor and probably oxidize it to carbon dioxide (15). Although not enough information is presently available to discuss the mechanism of the glycine fermentation by *D. glycinophilus* with authority, it seems likely that this organism uses glycine both as an oxidant and a reductant. It is still possible, however, that the decomposition of glycine may be another example of an "acetic acid fermentation" (16, 17) in which glycine is oxidized and carbon dioxide is reduced to acetic acid.

The formation of hydrogen from glycine has several features of special interest. Two mechanisms of hydrogen formation have been established with other microorganisms. Bacteria of the coli-aerogenes group form hydrogen by the decomposition of formate (18). *Cl. butylicum* and probably other butyric acid bacteria convert pyruvate to acetylphosphate, carbon dioxide and hydrogen (19). Formate is not an intermediate in this reaction. Hydrogen is also formed from a number of amino acids by *Cl. tetanomorphum* (20). This may involve still another mechanism of hydrogen formation, but it is equally probable that here also pyruvate is the immediate precursor of hydrogen. *Cl. tetanomorphum* forms hydrogen from pyruvate. In the glycine fermentation by *D. glycinophilus*, both formate and pyruvate are practically excluded as precursors of hydrogen since formate is not attacked at all and pyruvate is decomposed much too slowly. It must be concluded that a third, as yet unknown, mechanism of hydrogen formation is involved.

A distinctive feature of the hydrogen forming mechanism of *D. glycinophilus* is the great influence of hydrogen pressure on the yield of hydrogen. As the hydrogen partial pressure is increased from zero to 20% of an atmosphere the yield drops rapidly and above 25% no hydrogen accumulates. A somewhat similar but smaller effect of hydrogen was observed by Kubowitz (21) in the butyric acid fermentation of glucose by *Cl. butylicum*. With this organism, the presence of a hydrogen atmosphere changes the entire course of the fermentation

presumably by interfering with the oxidative decarboxylation of pyruvate. The result is that lactate is formed in place of acetate, butyrate, carbon dioxide and hydrogen. With *D. glycinophilus*, on the contrary, the only qualitative effect of a hydrogen atmosphere is to suppress hydrogen formation; the other products and the overall rate of glycine decomposition remain the same. This suggests that the formation of hydrogen is due to a readily reversible reaction (8a) which normally competes with a second reaction (8b) involving an organic hydrogen acceptor.



In a hydrogen atmosphere, reaction (8a) is suppressed in favor of reaction (8b). At the moment there is no evidence by which to identify the compounds  $AII_2$  and B.

### SUMMARY

Fermentations of amino acids and related compounds by two recently described anaerobic bacteria have been studied.

*Clostridium propionicum* causes a propionic acid type fermentation of alanine, serine, lactate, acrylate and pyruvate. Threonine is similarly decomposed except that butyric and propionic acids are formed instead of propionic and acetic acids. The butyric acid is probably formed by reduction of the four carbon chain in threonine rather than by condensation of two molecules of a  $C_2$  compound. Part of the threonine is oxidized to propionic acid.

*Diplococcus glycinophilus* causes a fermentation of glycine with formation of acetic acid, carbon dioxide, hydrogen and ammonia. No other simple compound is able to replace glycine as an energy source although several dipeptides containing a glycine residue with a free carboxyl group can be hydrolyzed. Benzoyl- and acetylglycine are also used. The yield of hydrogen and other products from glycine is dependent on the partial pressure of hydrogen. When the hydrogen partial pressure is above 25% of an atmosphere no hydrogen is evolved. Neither pyruvate nor formate can be the immediate precursor of hydrogen since pyruvate is metabolized much too slowly and formate is not decomposed at all.

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# Studies on the Metabolism of Some Ribose Derivatives

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## INTRODUCTION

It was observed that incubation of purine nucleotides and nucleosides with suspensions of minced tissues leads to a decrease in pentose concentration, as measured by the orcinol reaction (1). Since information concerning the metabolism of ribose is meager, it was tempting to pursue this lead. The following report deals with the conditions under which the reaction occurs, the distribution of the enzymes involved, and some observations on the products.

## EXPERIMENTAL

*Pentose Determinations.* For determination of pentose the orcinol reaction in the modification of W. Mejbaum was used (2). The samples were heated 40 minutes in a boiling water bath, instead of 20 minutes as recommended by Mejbaum. Purine nucleotides, nucleosides and pentose phosphates in equimolar solutions show slightly different color intensities ( $\pm 10\%$ ). For accurate results it would seem necessary, therefore, to use a reference standard identical to the unknown. This was impossible in our investigations, since mixtures of pentose derivatives had to be determined. *d*-Ribose was used as a reference standard. The samples, taken at times specified in the experiments, were treated with 2 volumes of 1 *N* HCl to stop enzymatic activity, and, after some standing in the cold, were diluted with water (1 to 50 or 1 to 100) to a suitable range for the pentose determination (5 to 25  $\gamma$ /sample). Small amounts of protein do not interfere, nor do purine bases (guanine, xanthine) influence the pentose reaction. In experiments with homogenized tissue the interference of excessive amounts of protein was eliminated by extracting the dye obtained in the orcinol reaction with amyl alcohol.

*Phosphate Determinations.* Fiske and Subbarow's method was used (3). The samples were deproteinized with trichloroacetic acid.

*Substrates.* The pentoses, adenosine-3'-phosphoric acid, guanylic acid and yeast nucleic acid were commercial products. Adenosine-5'-phosphate, inosinic acid, cozymase, guanosine, adenosine, ribose-3-phosphate and ribose-5-phosphate were pro-

TABLE I  
*Rate of Pentose Disappearance at Different pH Values*

pH	4.1	5.1	6.0	7.0	8.0	9.1
$\gamma$ -Moles of ribose disappeared after 3 hrs.	0.3	2.5	4.8	5.1	3.8	3.3

Enzyme: Dialyzed extract of acetone treated rabbit liver. Substrate: Guanosine (7.7  $\gamma$ -moles/ml.); pH adjusted with 0.1 *N* HCl and 0.1 *N* NaOH.

TABLE II  
*Distribution of Pentose Metabolizing Enzymes in Rat Tissues*

Tissue examined	Substrate	
	Guanosine	Ribose-5-phosphate
Liver	+++	+++
Kidney	+++	+++
Spleen	++	++
Brain	++	++
Muscle (hind leg)	+	
Blood	++	+
Testis	+	++
Heart	+	+

Suspensions of 285 mg. of homogenized fresh tissue/ml. were used. Concentration of substrates: 10 mg./ml. Incubation time: 3 hours.

TABLE III  
*Disappearance of Pentose from Purine Nucleotides and Nucleosides on Incubation with Rat Liver Suspension*

Substrate	$\gamma$ -Moles of pentose/ml.			
	0 hr.	1 hr.	3 hr.	5 hr.
No substrate	7.0	7.1	7.2	5.6
Adenosine-5-phosphate	20.0	12.7	10.4	9.5
Adenosine-3-phosphate	18.0	15.1	12.1	10.1
Cozymase	15.5	8.6	8.3	—
Inosine-5-phosphate	18.8	13.1	10.4	—
Guanylic acid	13.9	12.4	10.3	7.7
Ribonucleic acid	14.0	11.7	10.9	9.6

Rat liver suspension in 0.015 *M* phosphate buffer, pH 7.5; 285 mg. tissue/ml. incubation mixture.

pared according to the usual methods (4). Guanosine is the most readily available purine nucleoside and was used in many experiments. It has the disadvantage of being rather insoluble, which necessitates its being pipetted in suspension. Soon after mixing with the enzyme, however, it goes into solution.

*Enzymes.* Unless otherwise specified, liver of rat, rabbit, dog and pig was used.<sup>1</sup> In early experiments, suspensions (1 part tissue and 2.5 parts of 0.1 *M* phosphate buffer, pH 7.5) were prepared with a Waring blender. It was found that after centrifugation a considerable part of the enzymatic activity remains in the supernatant. These solutions were unstable. The preparation was improved by the use of acetone-dried liver. The fresh tissue was treated with 4 volumes of cold acetone in the Waring blender, filtered and the acetone treatment repeated. The resulting cake was broken up and the adherent acetone removed by reduced pressure in a desiccator. By this procedure a rabbit liver can be transformed into the dry product in 20–30 minutes. As it is essential to avoid delay, larger amounts of liver were divided into smaller portions, which could be handled rapidly. The dry product is stable for some days. In the experiments it was extracted with 6 times its weight of 0.1 *M* phosphate buffer (pH 7.5) at 37°C. for 30 minutes. After filtration and centrifugation a dark solution was obtained. By dialyzing for 12–24 hours against 0.02 *M* phosphate buffer of pH 7.5, the pentose content is lowered to a minimum, and the phosphate content is decreased. This eliminates high blank values in the determinations.

*Incubation mixture.* Incubations were carried out at 37°C. A small amount of toluene was added as a preservative. The concentration of substrate usually ranged from 5 to 15  $\gamma$ -moles/ml. The concentration of protein was 20–30 mg./ml. for experiments with extracts of acetone-dried tissue, and 40–60 mg./ml. for those in which homogenized tissue was used. Details are given in the Tables.

## RESULTS

The action of minced liver on adenosine and guanosine is illustrated in Fig. 1. The pentose disappearance is an enzymatic process. Heating of the tissue extracts or suspensions to 100°C. for 5 minutes destroys the activity. The rate of the reaction depends on the pH of the medium (Table I).

The distribution of the enzymatic activity in some tissues is shown in Table II. Liver was selected as a convenient source for the preparation of enzyme extracts in further studies. Table III shows results obtained with some purine nucleotides. It can be seen from the blank experiment that the pentose compounds present in the liver suspension itself are more stable than the pentose derivatives added as substrate. This may be due to the polymerized state in which the major part of the ribonucleotides is present in tissues. The disappearance of pentose

\* The authors are indebted to Dr. C. P. Coogle for help and a generous supply of tissues.



from nucleosides is not complete when fresh tissue suspensions are used, even after prolonged incubation (Table IV). This may be due to inactivation of the tissue enzymes before the reaction is completed.

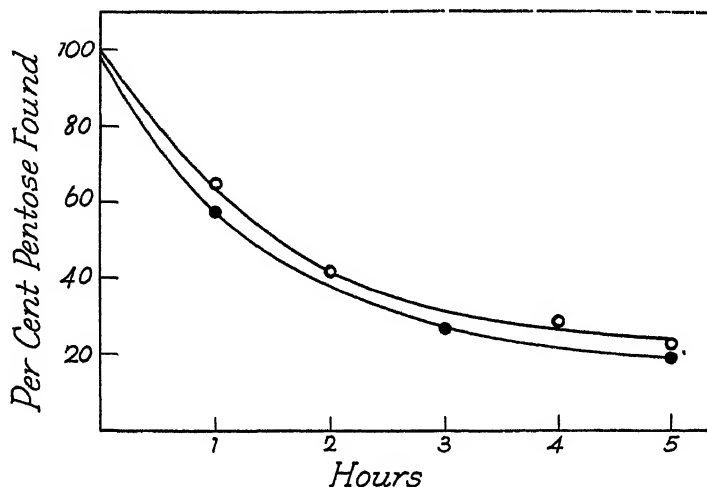


FIG. 1. Disappearance of Pentose from Adenosine (●—●—●) and Guanosine (○—○—○) upon Incubation with Homogenized Liver.

TABLE IV  
*Effect of Prolonged Incubation on Pentose Disappearance*

Substrate	$\gamma$ -Moles of pentose/ml.				
	0 hr.	2 hr.	6 hr.	13 hr.	24 hr.
No substrate	6.9	4.4	4.6	4.5	4.1
Adenosine	17.5	13.3	8.7	8.6	8.3
Guanosine	17.7	12.8	10.7	9.9	9.2

Enzyme: Rat liver suspension in 0.015 *M* phosphate buffer, pH 7.5; 285 mg./ml.

With extracts from acetone-dried liver the process can sometimes be brought to completion.

For elucidation of the phenomenon it would be desirable to have a substrate simpler than the nitrogenous derivatives of pentose. Unfortunately, *d*-ribose and other free pentoses are not affected by our enzyme preparations (Table V). This is in line with earlier experience

TABLE V  
*Incubation of Free Pentoses with Liver Enzyme*

Compound examined	$\gamma$ -Moles pentose/ml.		
	0 hr.	1 hr	3 hr
<i>d</i> -Ribose	10.3	10.0	9.3
<i>d</i> -Arabinose	11.3	12.6	11.5
<i>d</i> -Lyxose	12.2	11.4	11.0
<i>d</i> -Xylose	10.7	10.9	10.4
Guanosine	12.3	6.5	4.8
No substrate	2.4	2.3	2.3

Dialyzed enzyme extract from acetone-treated rabbit liver in 0.015 *M* phosphate buffer, pH 7.5.

TABLE VI  
*Effect of Oxygen on Pentose Disappearance*

Substrates and inhibitors	Hrs.	$\gamma$ -Moles of pentose/flask	Oxygen uptake, $\gamma$ -Moles/flask
No substrate	0	3.3	0
	5	2.7	2.1
Guanosine	0	25.8	0
	5	5.7	4.0
Guanosine+0.02 <i>M</i> iodoacetate	0	25.2	0
	5	6.0	0.7
Guanosine+0.02 <i>M</i> NaF	0	24.3	0
	5	5.4	2.7
Ribose-5-phosphate	0	26.7	0
	5	10.8	2.0
Ribose-5-phosphate+0.02 <i>M</i> iodoacetate	0	24.6	0
	5	11.7	1.0
Ribose-5-phosphate+0.02 <i>M</i> NaF	0	22.5	0
	5	12.3	2.6

Warburg technique;  $t=38^{\circ}\text{C}.$ ;  $\text{O}_2$ : 0.3 ml. of 10% KOH in center cup. 3.0 ml. solution containing substrate as specified; enzyme: dialyzed extract of acetone-treated rabbit liver, 46 mg. of protein/flask. Concentration of phosphate buffer: 0.013 *M*.

of others (5). It should be mentioned that the experiment reported in Table V furnishes proof that the disappearance of the pentose reaction is not due merely to gradual enzymatic formation of an inhibitor of the orcinol reaction. Likewise, purine bases added to the incubation mixture do not interfere with the reaction.

TABLE VII  
*The Role of Phosphate in Pentose Metabolism*

Exp. no.	Substrate and buffer	Hrs.	$\gamma$ -Moles of pentose/ml. found	$\gamma$ -Moles/ml. of inorganic phosphate found
1	Guanosine in 0.01 <i>M</i> phosphate buffer	0	8.5	11.0
		3	1.9	4.0
2	Guanosine in 0.02 <i>M</i> barbital buffer	0	9.1	—
		3	6.9	—
3	Guanosine in H <sub>2</sub> O	0	8.7	—
		3	8.2	—
4	Ribose-5-phosphate in H <sub>2</sub> O	0	7.5	—
		3	3.8	—
5	Guanosine in 0.045 <i>M</i> phosphate buffer	0	9.6	44.5
		3	1.5	36.0
6	Ribose-5-phosphate in 0.045 <i>M</i> phosphate buffer	0	7.6	45.1
		3	3.0	44.5

Enzyme: Extracts of acetone-treated rabbit liver were used. The solutions were dialyzed 16 hrs. for experiments 1 and 2; 40 hrs. for experiments 3 and 4; 48 hrs. for experiments 5 and 6.  $t=38^{\circ}\text{C}$ ;  $\text{pH}=7.5$ .

It was next determined whether oxygen is necessary for the reaction. Table VI shows that the oxygen uptake in the course of the reaction does not significantly exceed that of the blank experiment. Iodoacetic acid and sodium fluoride in the concentrations listed have no influence. It may be, however, that our enzyme extract is an incomplete system, and that, in a later stage of pentose degradation, oxidation plays a part.

In further experiments it was found that phosphate ion is necessary for the disappearance of pentose from nucleosides (Table VII). Phosphate ion has been found essential by W. Klein (6) for the activity of

nucleosidase. Since liver and kidney are rich in nucleosidase, we were led to believe that nucleosidase action is the first step in our system. Iodometric titrations verified this. Guanosine does not react with hypiodite under the conditions outlined for aldose determination (7). Free guanine, however, reacts with two molecules of  $I_2$ . This can be used to trace the liberation of the purine base from the nucleoside by nucleosidase action (8). Under our usual experimental conditions, using guanosine as substrate, we found 75% of the base liberated after 30 minutes as indicated by iodometric titration. H. M. Kalekar (9) has recently explained the role of phosphate in nucleosidase action. Phosphoric acid is bound in organic linkage and ribose-1-phosphate and base are products of enzymatic splitting of nucleosides. Although the properties of this new phosphate ester have not yet been described in detail, its lability toward acid has been emphasized. Fifty *per cent* destruction in 0.3 *N* HCl at 30°C. in 1 minute was observed by Kalekar.

The phosphate ester formed in our experiments is different from ribose-1-phosphate. It survives the deproteinization procedure and storage in acid for hours. Only by heating in 1 *N* acid at 100°C. is phosphate gradually split off.

We began to consider more stable phosphoric acid esters as intermediates. Incubation of ribose-5-phosphate with tissue enzymes was found to result in a decrease in pentose, while ribose-3-phosphate was almost inert (Table VIII).

TABLE VIII  
*Incubation of Ribose Phosphate with Liver Extract*

Compound examined	$\gamma$ -Moles of pentose/ml. found after		
	0 hr.	1 hr.	3 hr.
Ribose-5-phosphate	10.9	6.5	2.3
Ribose-3-phosphate	10.9	11.4	9.4

Enzyme: Phosphate buffer extract of acetone-treated rabbit liver. Blank values for pentose are subtracted.

It is apparent that ribose-5-phosphate shows the same disappearance of pentose as do nucleotides and nucleosides under the influence of tissue enzymes. For brevity, some experiments with this compound have been included in earlier tables. Aside from apparently identical distribution in tissues of the enzymes for both types of substrates

(Table II), the anaerobic nature of the process and its being insensitive toward fluoride and iodoacetate are shown in Table VI. The role of phosphate is less clear for the disappearance of pentose from ribose-5-phosphate. Experiments 4 and 6 in Table VII show that phosphate is not required. In other instances, however, we obtained extracts which bind some phosphate into organic linkage, while pentose disappears (Table IX). It would be tempting to explain the results given in

TABLE IX  
*Incubation of Ribose-5-Phosphate with Liver Extract*

Incubation time, hrs.	$\gamma$ -Moles of pentose found/ml.	$\gamma$ -Moles of phosphate found/ml.
0	9.8	12.1
$\frac{1}{2}$	4.6	10.7
1	4.1	9.6
2	3.6	9.1

Enzyme: Dialyzed extract of acetone-treated rabbit liver.

Table IX by assuming that a labile phosphate group bound by ribose-5-phosphate is partially hydrolyzed by the reagent of Fiske and SubbaRow (3). However, using Lowry's technique (10), which is better suited for determining labile phosphate esters, we found no difference.

Experiments using guanosine or adenosine as substrate were carried out on a large scale. After deproteinizing with trichloroacetic acid, the reaction products could be isolated by precipitating with  $\text{Hg}^{++}$  or  $\text{Pb}^{++}$ . The main fraction contained the purine base and a phosphoric acid ester. The barium salt of the latter was moderately soluble in water and was precipitated by the addition of alcohol. Some of its properties are listed here: Stability in acid: no phosphoric acid is liberated by 1.0 *N* HCl at room temperature after several hours. Heating in 1 *N*  $\text{H}_2\text{SO}_4$  at 100°C. splits off 15% phosphoric acid in 2 hours. Stability in alkali: 20% phosphoric acid is split off in 0.2 *N* NaOH at 100°C. after 3 minutes, and in 1 *N* NaOH at 20°C. after 15 minutes. The compound reduces hypiodite. The best preparations show an orcinol reaction corresponding to a pentose content of less than 2%.

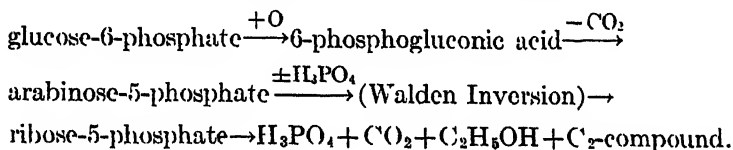
## DISCUSSION

Our knowledge of pentose metabolism has been advanced in recent years, particularly by F. F. Nord (11), F. Dickens (5, 12), W. Klein

(6) and H. M. Kalckar (9). The relation of our findings to their investigations will be discussed briefly.

In experiments with *Fusaria*, Nord and his group found that phosphorylation is not involved. Phosphoglyceric acid was not formed nor utilized by these organisms. This emphasizes the fact that the data reported above do not hold for all types of cells.

Dickens found that ribose-5-phosphate is fermented by yeast preparations, while free ribose and other pentoses are not. He assumed ribose-5-phosphate to be an intermediate in the degradation of hexose-6-monophosphate by oxidation and decarboxylation (13). The following explanation was given and in part verified:



Our findings on the role of phosphoric acid agree with those of Dickens. However, the fact that the yeast enzyme system needs nicotinamide nucleotides, and that it yields end products different from ours, shows that in that respect Dickens' results and our experiments are not related.

W. Klein found (6) that phosphate ion is required for the action of purine nucleosidase. H. Kalckar was able to explain this (9) by demonstrating that ribose-1-phosphate is formed in this process. The liberation of the purine base in our experiments proves that nucleosidase action is one of the steps involved. The stability of the linkage between phosphoric acid and carbohydrate in the product obtained by us excludes its identification with ribose-1-phosphate. Furthermore, our compound does not give the orcinol reaction, while Kalckar's ester responds to this reagent. To explain our observations, we must assume that ribose-1-phosphate undergoes further changes. At least two reactions occur: the stabilization of the phosphate group, and the rearrangement in the carbohydrate group responsible for the disappearance of the orcinol reaction. Our experiments with ribose-5-phosphate have led us to believe that this compound may be an intermediate in the sequence of reactions. A possible mechanism has been suggested in an earlier communication (1).

The isolation and nature of the reaction products and the enzymes involved will be studied further.

## SUMMARY

1. The ribose of nucleotides and nucleosides is changed by tissue enzymes to a product which does not respond to the orcinol test.
2. A stable phosphoric acid ester is formed in this process.
3. The conditions, under which the reaction takes place, are reported and the mechanism is discussed.

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## The Formation of Antimalarial Agents by Ultraviolet Decomposition of Quinine

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### INTRODUCTION

The decomposition which quinine undergoes when its solutions are exposed to ultraviolet light has been investigated and reported recently by Kyker, Cornatzer and McEwen (1). Analytical methods which depend on totally different structural properties of the alkaloidal nucleus were chosen for the characterization of the irradiated solutions. The selected procedures were applied to each of the solutions, both before and after extraction, under conditions which would remove any unchanged quinine. This report is a continuation on the nature of the photochemical degradation of the quinine molecule as revealed by the measurement of the biological activity of the products of irradiation before and after their partial fractionation. An attempt to correlate this activity with structural changes which may describe this decomposition is made.

Other investigators have reported on the biological activity of irradiated quinine. Macht and Teagarden (2) stated that ultraviolet light renders solutions of quinine more active pharmacologically. Conflicting reports have appeared regarding the effect of irradiated quinine on *Paramecium*. Various workers (3, 4, 5) observed that an increase in toxicity accompanied irradiation, while others (6, 7) observed no significant difference in irradiated and non-irradiated solutions with respect to their action on this organism. Fedorov (8) and Nitta (9) found that irradiation increased the action of quinine on

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infusoria and tissue cultures, respectively. The work of Roskin and Romanova (10) constitutes the only report which has dealt with the antimalarial activity of irradiated quinine. They found the activity of quinine on malaria to be enhanced considerably by irradiation. These observations were not accompanied, however, by any quantitative data which correlated the alkaloidal content of the solutions before and after irradiation with their biological activity.

## EXPERIMENTAL

### Method

Pekin ducklings from 2 to 4 weeks of age were used as the host for *Plasmodium Lophurae*, according to the method of Hewitt (11), for the antimalarial evaluation of each of the materials tested. The rate of infection was determined by counting the number of parasitized cells/500 red blood cells in Giemsa stained smears and was expressed as the percentage of parasitized cells. Each duckling which served as a donor had an infection of 30–40%. The volume of blood in each subject was estimated from its weight; and that volume of blood from a donor, whose rate of infection was known, was calculated to produce an initial infection of 2% upon injection into the former. This amount was taken from the donor by cardiac puncture, citrated and immediately injected into the host by way of the inner leg vein. Blood smears were taken daily for a period of 8 days.

The ducklings which were infected by an injection of parasitized blood were separated into 5 groups according to the treatment which was administered subsequently. The first group received no treatment (Table II). The second group received quinine (Table III). The third, fourth and fifth groups received materials which are designated as: Solution I (Table IV), Solution II (Table V), and Solution III (Table VI), respectively. Each of the ducklings which received treatment was assigned an individual dose on the basis of body weight in such manner that graded doses were represented within each group. All doses were administered daily by intraperitoneal injection.

TABLE I

Solution	pH	Apparent Concentration of Quinine in Per Cent		
		Turbidimetric Analyses		Fluorometric Analyses; Extractable Substances
		Unextracted Solution	Extractable Substances	
I	2.15	24.6	25.2	57.8
II	2.88	31.5	31.5	76.5
III	2.18	13.6		

Table I: Direct fluorometric determinations were necessarily omitted because of the quenching effect of the chloride ion which was present in the irradiated solutions.

TABLE II  
*Malarial Infection in Untreated Ducks*

Subject No.	Percentage Infection (Days after Inoculation)							
	1	2	3	4	5	6	7	8
1	1.5	5.6	8.5	21.4	52.8	37.5	6.3	1.5
2	3.9	9.0	22.4	45.2	51.6	48.8	17.0	
3	2.6	7.9	23.5	48.4	65.2	35.7	4.3	
4	2.4	5.1	14.6	23.0	46.5	42.9	13.8	4.0
5	2.3	5.0	16.0	34.6	49.2	35.9	29.8	25.9
Average	2.5	6.5	17.0	34.5	53.1	40.2	14.2	6.3

TABLE III  
*Malarial Infection in Ducks Treated with Quinine Dihydrochloride*

Dose: mg. free base/kg.	Subject No.	Percentage Infection (Days after Inoculation)							
		1	2	3	4	5	6	7	8
5.0	6	2.8	9.6	23.7	45.7	50.8	45.1	28.7	
7.5	7	2.8	4.8	5.3	12.7	21.7	18.6	8.1	3.2
	8	2.8	2.7	10.2	16.0	27.0	24.4	13.0	8.3
10.0	9	2.7	5.6	6.2	10.4	11.2	3.9	1.6	
	10	2.9	1.7	3.7	3.9	3.2	3.3	0.3	0.5
15.0	11	2.3	4.4	3.5	4.2	4.6	3.7	1.2	0.7
	12	2.9	3.7	4.2	5.0	4.2	2.1	0.9	1.1
20.0	13	3.9	6.6	5.5	7.2	5.6	3.4	0.7	
	14	2.5	1.6	2.1	2.0	1.7	0.8	0.4	0.1
50.0	15	0.6	0.1	0.1	0.0	0.0	0.0	0.0	0.0

#### *Materials*

The solutions which were employed in the tests may be described as follows:

Solution I resulted from the mixing of several solutions of quinine which differed only in their concentration of HCl and which had been exposed to sunlight for 2 years under conditions identical with those previously described for the exposure of similar solutions to sunlight for 275 days (1). The excess HCl which was present in

TABLE IV  
*Malarial Infection in Ducks Treated with Solution No. I*

Dose mg./kg.	Subject No.	Percentage Infection (Days after Inoculation)							
		1	2	3	4	5	6	7	8
5.00	16	2.7	10.4	19.1	50.0	59.4	35.8	10.3	
1.23	17	2.4	12.1	17.8	44.6	60.2	47.7	8.6	
1.26									
2.89									
7.50	18	3.2	5.3	14.7	17.5	43.7	29.9	21.0	7.5
1.84	19	1.9	4.6	13.3	32.3	53.3	37.7	4.9	3.3
1.89									
1.33									
10.00	20	2.4	9.9	14.7	36.1	49.9	38.6	8.5	
2.46	21	3.8	7.4	15.6	33.7	42.2	43.6	22.0	
2.53	22	2.1	5.1	9.1	13.5	26.1	38.1	33.8	29.8
5.78									
15.00	23	2.9	5.3	4.7	12.2	10.4	9.1	7.3	5.1
3.69	24	3.0	5.2	5.1	7.5	6.9	3.4	1.1	1.1
3.79									
8.67									
20.00	25	3.0	5.6	5.6	4.6	4.4	1.8	0.6	
4.92	26	3.8	5.7	5.7	4.8	5.0	2.9	2.0	
5.06	27	2.4	3.5	4.9	5.6	4.0	5.7	2.2	1.9
11.56									
50.00	28	0.9	0.1	0.1	0.0	0.0	0.0	0.0	0.0
24.80									
23.60									
—									
100.00	29	0.3	0.0	0.1	0.1	0.0	0.0		
50.00									
46.30									
—									

Table IV: The dose which each subject received is represented by 4 different values which are bracketed together. The first value expresses the amount of quinine which the same injection would have contained if the solution had never been irradiated. The second, third and fourth values are analytical results which were obtained respectively as follows: (a) by the turbidimetric analysis of the solution, (b) by the turbidimetric analysis of the ether extractable substances and (c) by the fluorometric

TABLE V

*Malarial Infection in Ducks Treated with Solution No. II*

Dose mg./kg.	Subject No.	Percentage Infection (Days after Inoculation)							
		1	2	3	4	5	6	7	8
10.00	30	3.0	3.9	8.4	13.7	11.2	9.0	6.9	5.1
3.15	31	1.5	6.5	9.4	21.4	22.2	18.4	9.0	4.0
3.15									
7.65									
15.00	32	2.0	4.4	7.1	4.7	5.0	1.6	0.6	0.0
4.72	33	2.8	7.6	11.3	16.5	12.8	5.5	5.9	2.7
4.72									
11.47									
20.00	34	2.7	4.8	3.9	4.5	2.7	0.9	0.3	0.1
6.30	35	2.2	5.4	5.5	8.0	4.3	1.8	0.5	0.5
6.30									
15.31									

Table V: The significance of the 4 values for each dose in this table is the same as that which is explained for Table IV.

TABLE VI

*Malarial Infection in Ducks Treated with Solution No. III*

Dose mg./kg.	Subject No.	Percentage Infection (Days after Inoculation)							
		1	2	3	4	5	6	7	8
15.00	36	2.6	3.7	10.3	12.1	16.9	15.5	10.9	7.0
2.04	37	1.9	4.2	13.0	24.5	36.6	36.5	26.8	18.1
20.00	38	1.6	5.4	8.5	9.4	9.2	2.5	4.1	0.7
2.72	39	2.5	3.9	11.5	17.9	22.5	25.4	13.0	8.8
50.00	40	2.5	4.8	3.7	5.9	3.0	1.7	1.1	1.3
6.80	41	2.4	2.4	2.8	4.2	2.0	1.4	0.5	0.2

Table VI: The 2 values for each dose have the same significance as the first and second values for each dose in Tables IV and V.

the mixture was neutralized with NaOH until a trace of a flocculent precipitate appeared. The precipitate was dissolved by a slight addition of acid. The dilution which occurred in this adjustment of acidity was measured, and the concentration of the diluted solution was corrected accordingly.

Solution II was obtained by a procedure similar to that for Solution I except that an original solution of quinine in 2 M HCl was irradiated for 48 hours with a mercury arc lamp instead of sunlight (1).

Solution III was prepared by taking a portion of Solution I, rendering it strongly alkaline and extracting it continuously with ether for 20 hours. The apparatus and conditions for the continuous extraction were previously standardized by repeatedly showing more quinine to be removed completely in 12 hours from an equal volume of solution than the specified portion of Solution I contained before irradiation. The extractor was similar in construction to one of an earlier description (12) except that its dimensions accommodated a 50 cc. sample. Both the aqueous layer and its precipitate which formed when alkali was added in excess and which largely remained during the extraction were recovered from the apparatus by dissolving and rinsing with dilute HCl. The acidity was adjusted, as in the case of Solutions I and II, to a point which barely dissolved the solid.

The pH of each of the solutions was determined by measurement with a glass electrode and the content of the solutions was compared by different analytical methods which measure quinine (1). The characteristics of the 3 solutions are summarized in Table I. The analytical results are expressed as the apparent concentration of quinine since they represent both the unchanged quinine and all determinable substances which are products of photodecomposition and which respond to the methods for quinine. The results are calculated as the percentage of the original alkaloidal content before irradiation.

## RESULTS

*Untreated Subjects.* The data in Table II show for each untreated subject a rapid increase of *Plasmodium Lophurae* through the fifth day, followed by a similar decrease. The average maximal infection exceeded 50%. These results agree with those of Hewitt (11).

*Subjects Treated with Quinine.* The results in Table III indicate that 7.5 mg. of quinine/kg. of body weight is the minimal effective dose, whereas 10 mg./kg. strongly represses the increase of *Plasmodium Lophurae* in ducklings. Further suppression of the infections parallels increased doses, but the drug declines rapidly in efficiency beyond 15 mg./kg. These results agree quantitatively with those of Seller, Dusenbery and Malanga (13).

*Subjects Treated with Irradiated Solutions of Quinine.* Subjects 9-10, 23-24 and 32-33 (Tables III-V) were equally protected. These results compare quantitatively Solutions I and II with quinine. These solutions were administered at levels of 15 mg. of quinine/kg. before

irradiation. By turbidimetric analysis after irradiation, their content of quinine was equivalent to maximal doses of 3.69 and 4.72 mg./kg., respectively. From Table III, the minimal effective dose of quinine is twice the maximal amount which was provided by Solutions I and II. Similarly, a comparison of subjects 25-27 and 34-35 with subjects 11-12 reveals almost identical protection of the subjects. Furthermore, the actual quinine in Solution I is only half of the above maximum since Table I shows that Solution III retains approximately 50% of this analyzed fraction of Solution I. The same is concluded for Solution II because of the similar origin and properties of Solutions I and II.

The treatment of subjects 36-41 (Table VI) with Solution III, which by its preparation contains no quinine, establishes the antimalarial activity of the products of irradiated quinine. Subjects 40-41 received greater protection from Solution III than 20 mg. of quinine/kg. provides.

The above comparisons, and others which are apparent from Tables III-VI, indicate quantitatively that solutions of quinine retain at least two-thirds of their antimalarial activity after two-thirds of the alkaloid is destroyed. It follows, therefore, that these irradiated solutions contain an active antimalarial agent other than quinine.

## DISCUSSION

Neutralization of the irradiated solutions of quinine to a pH ordinarily desired for parenteral administration was impracticable since the resulting flocculation could not be injected quantitatively. No recognizable injury appeared from the intraperitoneal administration of these acid solutions.

Differences between quinine and its products of irradiation may be noted. The latter is less soluble in water above pH 2.5, and the flocculent solid which persists above this pH fails to extract with ether from strongly alkaline aqueous layers. These properties suggest an amphoteric nature and indicate that the basic ionization constant is smaller than that which characterizes quinine.

The excellent agreement of the third and fourth columns of Table I for Solutions I and II reflects an apparent discrepancy for the turbidimetric method (12) since Solution III represents substances in Solution I which appear unextractable by the same solvent. The preparation of Solution III from I employed conditions which were rigidly

standardized to remove more quinine than Solution I contained at any time before irradiation. Relatively concentrated solutions were employed in this preparation of Solution III for convenience in its later use. To meet the range of the turbidimetric method, aliquots were analyzed which had been diluted approximately 1,000 times. In the analytical procedure (12), there is sufficient margin for the complete extraction of micro amounts of the same product which appears unextractable when Solution III is in preparation.

An absolute comparison of the antimalarial activity of quinine and its products of irradiation cannot be made since both the chemical nature of the latter is unknown at present and no absolutely specific analytical tool exists for the former. Certain interpretations can be made, which follow.

The complex nature of the quinine molecule and of photochemical reactions in general presumes a complex mixture of products. Preliminary studies support this assumption. Pronounced changes in color during the irradiation precede any analytically detectable change (1). It is improbable that the irradiation produces a high yield of one substance or that the specific antimalarial nature of quinine would be retained by each if many substances are derived.

Less than one-sixth of the alkaloid remains undecomposed since the apparent maximum by analysis is 25-31%, nearly half of which constitutes Solution III, which is free of quinine. Therefore, the products of irradiation are approximately one-fifth as responsive as quinine to silicotungstate turbidimetry since products from five-sixths appear analytically equal to one-sixth. Similar reasoning indicates that the products are more than half as fluorescent as quinine. Antimalarial activity decreases less than fluorescence. Since neither of the 3 methods is totally specific for quinine, turbidimetry, which indicates the lowest maximum, is best for determining the extent of chemical change and for evaluating the antimalarial activity of the products. Accordingly, the data indicate that the products of irradiation include a substance which compares favorably with quinine and which may be more active biologically.

An attempt to correlate the turbidimetric, fluorometric and biological characteristics of the products of irradiation with the limited structural changes which quinine can undergo without loss of biological activity (15) renders impossible a proposal of the structural properties of the biologically active product. It is unique that the 3

methods for measuring quinine in this study yield quantitative results for its products in the reverse order of magnitude to that anticipated by their specificity.

#### ACKNOWLEDGMENT

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#### SUMMARY

Solutions of quinine have been irradiated under specified conditions with natural and artificial sources of ultraviolet. The products of the reaction were tested by chemical, physical, and biological procedures before and after partial fractionation.

The data reveal certain contrasting properties of quinine with its products of photodecomposition and prove that there is among the latter an effective antimalarial agent. The retention of the chemotherapeutic properties of quinine by its products lends added significance to the chemical nature of the reaction.

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# Phosphorylated Amino Acids \*

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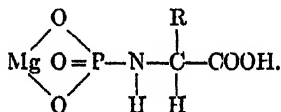
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## INTRODUCTION

In addition to the several types of biologically important phosphoric acid esters of organic compounds, two phosphoamides, phosphocreatine and phosphoarginine have been well characterized. In these latter substances the phosphorus is attached directly to a (guanidine) nitrogen atom.

More than thirty years ago Neuberg and Oertel (1) described a method for the synthesis of phosphoamino acids, compounds which resembled phosphocreatine in possessing phosphorus-nitrogen bonds. Glycine, alanine and tyrosine were phosphorylated by the action of phosphorus oxychloride on these amino acids in aqueous solution in the presence of excess magnesium oxide. Neuberg and Oertel also phosphorylated proteins and found that the products were digested readily by proteolytic enzymes. Subsequent studies of phosphorylated proteins are those of Rimington (2) and Mayer and Heidelberger (3). The last two investigators concluded that both amino and hydroxyl groups in proteins were phosphorylated by phosphorus oxychloride.

The phosphoamino acids prepared by Neuberg and Oertel were considered to be secondary magnesium salts of the structure



However, the analytical values for both nitrogen and phosphorus were much too low, and the magnesium values incorrect, for the postulated structures of the glycine and alanine derivatives. In the case of tyrosine, a small yield of phosphorylated product was obtained which analyzed correctly as a double secondary magnesium salt, i.e., both the hydroxyl and amino groups were phosphorylated.

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In view of the inadequate knowledge of phosphoamino acids, and of their possible significance in biological systems, the present study was undertaken with the purpose of repeating the preparative methods of Neuberg and Oertel, and of studying certain of the properties of the phosphoamino compounds.

### PREPARATION OF PHOSPHOAMINO ACIDS

The procedure employed was essentially that of Neuberg and Oertel. It will be described for the case of glycine to facilitate the discussion of present observations.

*Phosphoglycine.* Ten g. of glycine (0.133 mol) were dissolved in 200 ml. of water in a wide mouth bottle. Forty g. of magnesium oxide powder (1.0 mol) were added, and the mixture chilled to 0–5°C. in an ice bath. A solution of 25 g. of phosphorus oxychloride (0.16 mol) in 150 cc. of carbon tetrachloride was added over a period of 3 hours with efficient cooling and stirring. The pH remained in the vicinity of 8 during the reaction. After one additional hour of stirring at 5–10°C., the mixture was filtered with suction. The precipitate, which consisted of excess magnesium oxide and magnesium phosphate, was discarded.

After separating and discarding the carbon tetrachloride phase, the clear filtrate was adjusted to pH 7.0 with dilute acetic acid. The solution was chilled, and then 2 volumes of cold alcohol were added slowly with stirring. The resulting heavy, flocculent precipitate was collected with suction. Magnesium chloride and unreacted glycine remained in the filtrate.

To facilitate subsequent redissolving it was found necessary to remove the alcohol from the precipitate. The latter was washed in succession with 60%, 95% and absolute alcohol, and lastly with absolute ether. The resulting amorphous white powder was then placed for several hours or overnight in a vacuum desiccator over sulfuric acid to remove the ether.

The crude magnesium salt of phosphoglycine dissolved almost completely in about 250 ml. of water. After filtration, the clear solution was chilled, treated with 2 volumes of ice-cold alcohol and the resulting precipitate collected. Upon treatment with 95%, and then with absolute, alcohol, the precipitate became granular, although no definite crystals could be observed. The product was washed with absolute ether and dried in a vacuum desiccator over sulfuric acid. The yield was 10–12 g., or about 50% of the theoretical amount.

*Phosphoalanine and Phosphoglutamic Acid.* The procedures employed were similar to the foregoing. However, the yields were somewhat lower, generally 5–7 g. of phosphorylated product from 10 g. of *dl*-alanine or *l*-(+)-glutamic acid.<sup>1</sup>

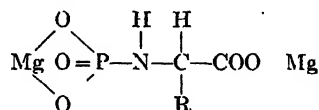
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<sup>1</sup> In other experiments, phosphorylated leucine and phenylalanine could be obtained only in rather low yields, owing apparently to the insolubility of the magnesium salts. Phosphorylated glycylglycine preparations invariably contained considerable unreacted glycylglycine, since the latter substance crystallizes easily from aqueous solution on the addition of alcohol.

## CHEMICAL ANALYSIS AND GENERAL PROPERTIES

*Elementary Composition and Structure*

Analysis indicated the presence of nitrogen and phosphorus atoms in one to one ratio in the 3 phospho compounds. However, 1.5 atoms of magnesium were found per atom of nitrogen or phosphorus in the phosphoglycine<sup>2</sup> and phosphoalanine preparations, while the phosphoglutamic acid salt contained two atoms of magnesium. Accordingly, the first two compounds appeared to be magnesium salts of the type



while in phosphoglutamic acid, both carboxyl groups were combined with magnesium.

TABLE I

*Elementary Composition of Phosphoamino Acids*<sup>1</sup>

Constituent	Phosphoglycine		Phosphoalanine		Phosphoglutamic acid	
	Calculated <sup>1,4</sup>	Found	Calculated <sup>1,4</sup>	Found	Calculated <sup>1,4</sup>	Found
Carbon	9.8	9.8-10.4	14.0	13.5	17.6	18.0
Hydrogen	3.7	2.4- 2.7	4.2	3.7	4.1	4.3
Nitrogen	5.7	5.4- 5.7	5.4	5.3- 5.6	4.1	4.0- 4.2
Phosphorus	12.7	12.4-13.5	12.0	11.2-12.3	9.1	8.7- 9.3
Magnesium <sup>†</sup>	14.9	15.4	14.2	13.8	14.2	12.7
Ash	53.7	54.9	51.0	49.8	44.3	40.9-42.0

<sup>1</sup> Nitrogen analyses were performed by micro Kjeldahl and phosphorus by the method of Fiske and Subbarow. Carbon and hydrogen determinations were performed by Mr. G. L. Stragand.

<sup>4</sup> For a trihydrate of the type,  $\text{MgO}_2\text{PNHCH}(\text{COO}(\text{Mg})) \cdot 3\text{H}_2\text{O}$ .

<sup>†</sup> For a tetrahydrate of the form,  $\text{MgO}_2\text{PNHCH}(\text{COO})(\text{CH}_2\text{CH}_2(\text{COO}))\text{Mg} \cdot 4\text{H}_2\text{O}$ .

<sup>‡</sup> Calculated from the phosphorus and ash determinations, assuming the ash to represent a mixture of  $\text{Mg}_3\text{P}_2\text{O}_7$  and  $\text{MgO}$ .

As is indicated in Table I, satisfactory agreement between analytical and theoretical values could only be obtained by assuming a considerable degree of hydration of the compounds, although the analyses were

<sup>2</sup> The calcium salt of phosphoglycine similarly had 1.5 atoms of calcium/atom of phosphorus.

performed on preparations dried as described above. The composition of the preparations was not markedly altered by additional precipitations from aqueous alcohol solution.

### *Amino Nitrogen*

From analyses in the Van Slyke deaminizing apparatus, it was found that from 10–25% of the total nitrogen of the phosphoamino acids reacted as free amino nitrogen in 4 minutes. These amino nitrogen values increased with longer periods of reaction, due presumably to hydrolysis of phosphorus-nitrogen linkages. When solutions of the compounds were treated with 1 *N* hydrochloric acid, 92–98% of the total nitrogen then reacted in 4 minutes.

The phospho compounds gave positive color tests with ninhydrin (in aqueous pyridine solution). The purple colors developed at a slightly slower rate (at 100°C.) than in parallel tests with free amino acids.

### *Physical Properties*

When kept free from moisture and in the refrigerator, the compounds were stable for several weeks. They dissolved readily in water, leaving no insoluble residue. The solutions were slightly alkaline, about pH 7.5 to 8.0. They remained clear for several hours at room temperature, but gradually became cloudy on longer standing. Heavy amorphous inorganic precipitates resulted when the solutions were heated.

### *Rates of Decomposition in Acid Solution*

Like phosphocreatine, the phosphoamino acids were rapidly hydrolyzed by dilute acid. The rates of hydrolysis at pH 3.0 could be conveniently and accurately determined by spectrophotometric measurements, since the phosphoamino acids were found to absorb ultraviolet light much more strongly than did the corresponding free amino acids plus inorganic phosphate. For purposes of comparison, the hydrolysis of phosphocreatine was also determined spectrophotometrically. The phosphocreatine was synthesized by the method of Zeile and Fawaz (4). It analyzed correctly as a secondary calcium salt.

The rates of decrease in the optical densities of the solutions were measured in the Beckman quartz spectrophotometer at 224  $m\mu$  and 25°C.<sup>3</sup> The difference,  $d$ , between the optical density at time,  $t$ , and at

<sup>3</sup> The relative decreases in optical density were greatest with phosphocreatine and phosphoalanine. The final values in these cases were about 45–50% of the initial readings.

infinite time, was proportional to the phosphoamide concentration. Plots of  $\log d$  against reaction time gave straight lines. For the first order equation, the velocity constant,  $k$ , equaled 2.303 times the slopes of these lines. The following were the constants obtained: phosphocreatine, 0.015; phosphoalanine, 0.029; phosphoglutamic acid, 0.029; and phosphoglycine, 0.059.

These values indicate that phosphocreatine was the most stable of the compounds tested, while phosphoglycine hydrolyzed most rapidly. The phosphoamino acid solutions were 0.005–0.01  $M$ , while phosphocreatine was employed at a 0.001  $M$  concentration (because of its stronger absorption at 224  $m\mu$ ). The hydrolysis constant calculated for phosphocreatine is somewhat larger than the value reported by Fiske and Subbarow (5), who followed the hydrolysis by measuring the liberated inorganic phosphate.

#### *Catalytic Effect of Molybdate on the Rates of Acid Hydrolysis*

The phosphoamino acids appeared to be far more sensitive to molybdate ion than was phosphocreatine (6). The relative rates of hydrolysis in the presence of 0.08% ammonium molybdate at pH 4.1, as measured by the liberation of inorganic phosphate, were determined according to the colorimetric procedure of Lowry and Lopez (6).

In Fig. 1 it is seen that the initial rates of color development by the phosphoamino acid solutions were only slightly less than the rate for inorganic phosphate and far more rapid than in the case of phosphocreatine. However, there were marked lags in the times required for maximum color development with phosphoamino acids as compared with inorganic phosphate. On the basis of these observations, the colorimetric method appears unsuitable for the measurement of the rates of hydrolysis of the phosphoamino acids.

#### *Heat Liberation upon Acid Hydrolysis*

Calorimetric measurements were employed to determine the phosphate bond energy of phosphoglycine. The following is a typical experiment.

A simple calorimeter, consisting essentially of a Dewar flask, a Beckman thermometer, and a stirrer, was calibrated by observing the temperature rise when 45 ml. of 0.0935  $N$  sodium hydroxide were neutralized by a slight excess of acid. The acid (7.5 ml. of 1  $N$  hydro-

chloric acid) was contained in a thin-walled sealed glass bulb. The latter was broken by the stirrer after an initial equilibrium temperature was established. The observed temperature increase was  $0.77^{\circ}\text{C}$ . (read after 20 minutes). Taking 58 calories as the theoretical heat of neutralization of the 4.20 milliequivalent of alkali, and 0.5 calorie as the heat of dilution of the acid, the heat capacity of the system was  $58.5/0.77$  or 76 cal./degree observed rise in temperature.

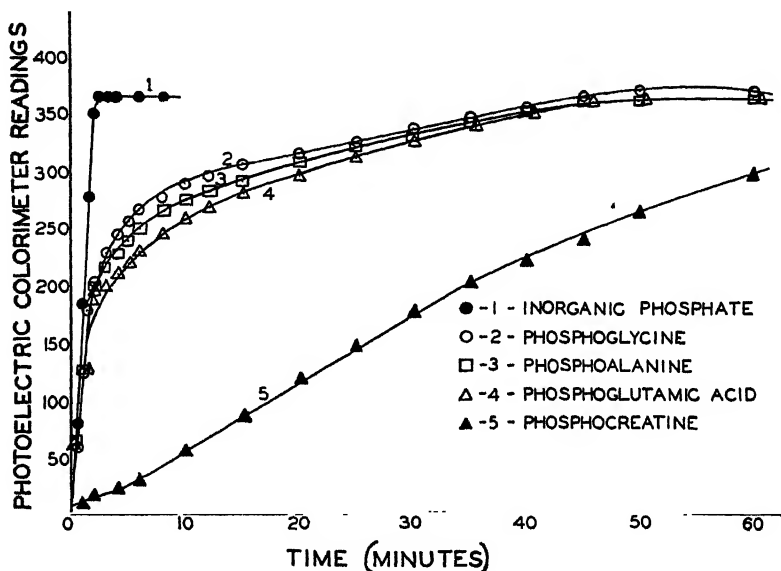


Fig. 1. Relative Rates of Hydrolysis of Phosphoamides in the Presence of Molybdate Ion. In each case the quantity of compound tested contained 55–60  $\gamma$  of P.

Next, 45 ml. of a solution of 1.30 g. of phosphoglycine (0.005 mole), adjusted to pH 5.0, were mixed as before with 7.5 ml. of 1 *N* acid. A maximum rise in temperature of  $0.51^{\circ}\text{C}$ . was observed after 20 minutes. Accordingly, the total calories liberated were  $0.51 \times 76$  or 38.5, and the molar heat of hydrolysis was  $38.5/0.005$  or, approximately, 7,500 calories. The final pH of the system was 2.5.

The molar thermal value found for phosphoglycine is smaller than that for the high energy phosphorus-nitrogen bond of phosphocreatine ( $\Delta H = 11,000$  cal.), but approximately equal to that of phosphoarginine ( $\Delta H = 8,000$  cal.) (7).

*Hydrolysis by Phosphatase*

In view of the finding that phosphocreatine is hydrolyzed readily by purified phosphatase preparations (8), it appeared of interest to determine whether or not phosphoamino acids were similarly split.

Inasmuch as the decomposition of the phosphoamides in neutral or alkaline solution yielded precipitates or cloudy solutions, the spectrophotometric method could not be employed. Also the instability of the

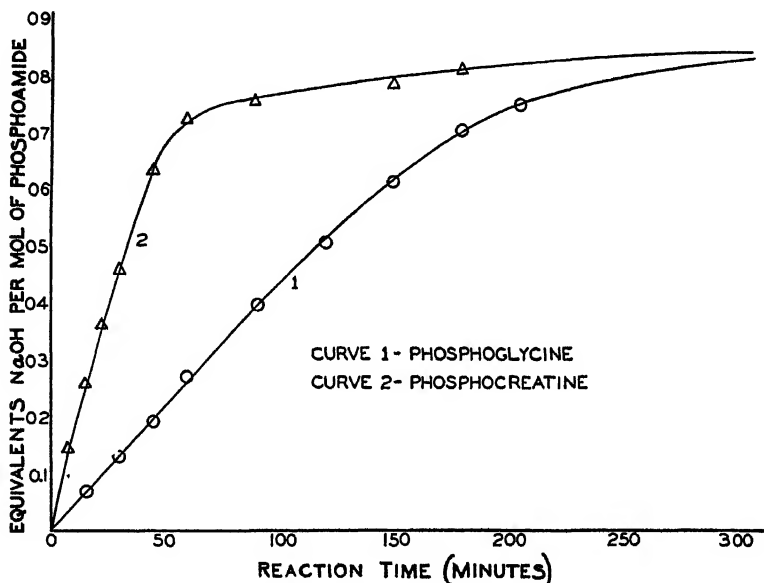


FIG. 2. Hydrolysis of Phosphoglycine and Phosphocreatine by Phosphatase as Measured by the Liberation of Acid During the Reaction. 250 mg. of each phosphoamide compound in 25 ml. of 0.005 *M* borate buffer of pH 9.0 were reacted at 25°C. with 5 ml. of phosphatase solution (pH 9.0) containing 0.08 mg. protein N/ml. The titrations (with 0.1 *N* NaOH) were corrected for small blanks, in parallel runs with inactivated enzyme.

compounds made difficult the measurement of free amino groups by the nitrous acid method or inorganic phosphate by colorimetry.

It was found, however, that the hydrolysis of the phosphoamides resulted in a shift in pH in the acid direction. For instance, the pH of a 0.005 *M* solution of the calcium salt of phosphocreatine changed from 8.15 to 5.65 when the solution was heated briefly at 100°C. This observation provided the basis for a method of measuring the hydroly-



sis of phosphoamides by alkaline phosphatase. By conducting the enzymic reaction in a weakly buffered solution initially adjusted to pH 9.0, and measuring at frequent time intervals the quantities of standard alkali required to bring the alkalinity of the solution back to pH 9.0, a measure could be obtained of the rate of liberation of acid groups.

This method gave best results with phosphoglycine. Phosphoalanine and phosphoglutamic acid were somewhat unstable at pH 9.0. Fig. 2 compares the rates of hydrolysis of phosphoglycine and phosphocreatine by partially purified hog intestinal phosphatase. This enzyme was prepared by the method described by Schmidt and Thannhauser (9). The initial rate of hydrolysis of phosphoglycine was approximately one-fourth as great as that of phosphocreatine. In both cases about 0.86 equivalent of acid was ultimately liberated per mol of compound.

#### SUMMARY

Three phosphoamino acids, analogous to phosphocreatine in structure, were prepared by the action of phosphorus oxychloride on glycine, alanine and glutamic acid in aqueous magnesia solution. The products were isolated as magnesium salts, containing nitrogen and phosphorus in equivalent proportions.

The phosphoamino acids were hydrolyzed somewhat more rapidly by dilute acid than was phosphocreatine. The hydrolyses resulted in the liberation of free amino nitrogen. The rates of hydrolysis were measured most conveniently by changes in the ultraviolet absorption spectra. Molybdate ion strongly accelerated the rates of decomposition.

In the case of phosphoglycine, the hydrolysis with acid was accompanied by a heat evolution of approximately 7,500 calories per mol. This compound was found to be hydrolyzed by phosphatase enzyme.

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# The Hydrolysis of Phosphocreatine by Phosphatase\*

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## INTRODUCTION

In 1933 Waldschmidt-Leitz and Köhler (1) observed that alkaline kidney extracts could hydrolyze phosphocreatine, as well as various phosphoric acid esters. But inasmuch as the activity toward phosphocreatine appeared to be independent of the phosphatase activity in the course of purification experiments, these investigators concluded that phosphoamides were hydrolyzed by a distinct enzyme, termed *phosphoamidase*. The same conclusion was reached by Ichihara (2), who employed as substrates synthetic phosphoamides, such as phosphoric acid anilide.

In view of the conjectural character of phosphoamidase, it appeared of interest to the author to test the action of phosphatases from different sources and of different degree of purity on phosphocreatine. The recent analytical procedure of Lowry and Lopez (3) makes possible the accurate measurement of inorganic phosphorus liberated from phosphocreatine. Disodium phenyl phosphate was selected as a reference substrate for the measurement of phosphatase activity.

## EXPERIMENTAL

*Enzymes.* Four alkaline phosphatase preparations were employed: (1) Crude, dialyzed aqueous extract of rat kidney; (2) partially purified phosphatase of hog intestines; (3) partially purified hog kidney phosphatase; and (4) a highly purified enzyme from calf intestines.<sup>1</sup> The second and third enzyme preparations were obtained by essentially the same method employed by Schmidt and Thannhauser (4) for the isolation of the calf intestinal phosphatase, but did not have quite as high an activity as the latter enzyme.

In the assay procedure, the crude rat kidney extract was employed at a concentration of 1.6 mg., the two partially purified hog tissue preparations at 0.035 mg., and the highly purified preparation at 0.015 mg. of protein nitrogen per ml. of enzyme solution.

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<sup>1</sup> Kindly supplied by Dr. Gerhard Schmidt.

*Substrates.* The enzymes were tested on both phosphocreatine and disodium phenyl phosphate. The phosphocreatine was synthesized by the method of Zeile and Fawaz (5). It analyzed correctly as a secondary calcium salt. Both substrates were employed as 0.5% solutions in 0.01 *M* borate buffer of pH 9.0.

*Method of Assay.* One ml. portions of enzyme solution (pH 9.0) were added to 1 ml. samples of substrate at 25°C. After varying intervals of time the reactions were stopped by the addition of 2 ml. of 5% trichloroacetic acid.<sup>2</sup> The quantity of inorganic phosphate formed was determined by colorimetric analysis of 1 ml. aliquots of the mixtures according to the method of Lowry and Lopez (3). The color readings were taken after exactly 3 minutes when phosphocreatine was employed, and after 6 minutes, or longer, when phenyl phosphate was the substrate. The phosphorus values were corrected for small blanks obtained in determinations with inactivated enzyme.

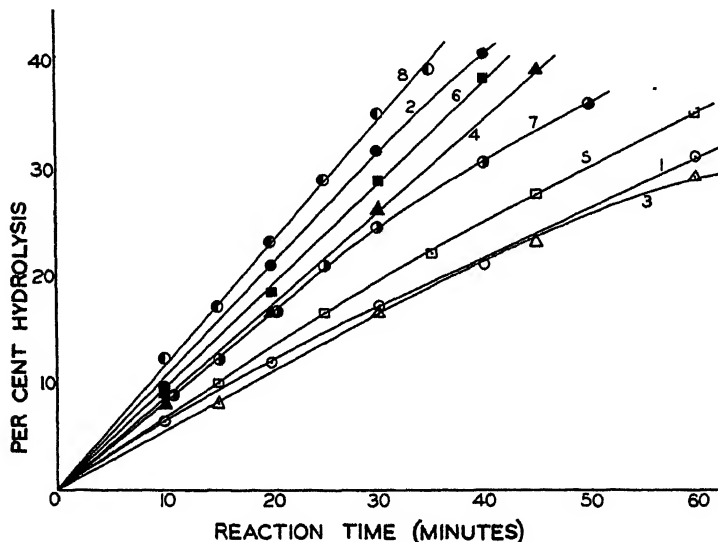


FIG. 1. Hydrolysis of Phosphocreatine and Disodium Phenyl Phosphate by Phosphatase Preparations

Curves 1 and 2 (open and solid circles), crude rat kidney extract on phosphocreatine and phenyl phosphate; Curves 3 and 4 (open and solid triangles), partially purified hog intestinal phosphatase on phosphocreatine and phenyl phosphate; Curves 5 and 6 (open and solid squares), partially purified hog kidney phosphatase on phosphocreatine and phenyl phosphate; and Curves 7 and 8 (half circles), highly purified calf intestinal phosphatase on phosphocreatine and phenyl phosphate, respectively.

<sup>2</sup> Filtration was necessary when the crude kidney extract was employed. When phosphocreatine was the substrate, ice-cold trichloroacetic acid was used and aliquots were rapidly delivered into buffer solutions to minimize acid hydrolysis.

## RESULTS

The rates of hydrolysis of phosphocreatine and of disodium phenyl phosphate by the four different phosphatase preparations are given in Fig. 1. Of significance, is the finding that the ratio of the rate of hydrolysis of phosphocreatine to that of phenyl phosphate does not appear to vary significantly. This ratio, as obtained from the initial slopes of the curves, is 0.57 for crude rat kidney extract, 0.62 for partially purified hog intestinal phosphatase, 0.70 for partially purified hog kidney phosphatase, and 0.72 for highly purified calf intestinal phosphatase.

Two further observations which may be mentioned are that both substrates were hydrolyzed most rapidly in the vicinity of pH 9.0 to 9.5 and that the addition of magnesium ion (0.02 *M* concentration) activated both reactions to the extent of about 20-30% for all 4 enzyme preparations. Schmidt and Thannhauser found a slightly lesser degree of activation by magnesium. Their purified enzyme attacked a variety of phosphorylated compounds, but phosphocreatine was not among the substrates tested.

In conclusion the present data do not support the view that phosphoamides are hydrolyzed by a specific enzyme distinct from alkaline phosphatase.

## SUMMARY

Phosphocreatine was found to be hydrolyzed readily by alkaline phosphatases from different sources and of varying degrees of purity. The ratios of the rates of hydrolysis of phosphocreatine and of disodium phenyl phosphate did not appear to vary significantly with the different enzyme preparations. Magnesium activated the splitting of both substrates.

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# Growth, Reproduction and Lactation in the Rat Maintained on Purified Diets \*

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## INTRODUCTION

During the past few years several groups of workers have reported on growth, reproduction and lactation in the rat maintained on purified diets. This work has been reviewed by Vinson and Cerecedo (1). The best results have been obtained by Vinson and Cerecedo (1, 2), and by Richardson and Hogan (3), both groups reporting growth and reproduction equivalent to that produced by stock diets but subnormal lactation. Lactation efficiency, as judged by the percentage of young weaned, was 60-70%.

Generation studies of rats maintained on purified diets have been carried out in the laboratory since 1941. This paper will report on growth and reproduction in both sexes and lactation in the female. The inadequacies of purified "control" diets used for short-term B vitamin studies have long been recognized. Our attempts at improvement finally led to the formulation of a new basal diet containing an improved salt mixture and supplemented with 8 crystalline B vitamins. This diet proved equivalent to the stock diet for the growth of weanling rats during the first generation and for their reproduction; although lactation was greatly improved, it was still not optimal.

## EXPERIMENTAL

Rats of the Long-Evans strain were used in all experiments. In first generation studies (unless otherwise noted) stock litters were placed on the experimental diets at weaning (21 days of age). The litters were divided into equivalent groups, *i.e.*, one littermate was placed in each dietary group and the groups balanced according

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TABLE I  
Composition of Purified Diets

Diet Number	s20	s22	s30	s31	s33	s37	s38
Casein	24	21	25[1]	24	24	64	24
Sucrose	62	61	62	64	61	24	21
Hydrogenated Vegetable Oil (Crisco)	8	8	8	8	8	8	48
Cod Liver Oil	2	2					
Fat-soluble Vitamin Mixture [1]	[3]	[3]	0.5 cc	0.5 cc	0.5 cc	0.5 cc	0.5 cc
Salts [2]	4(A)	5(B)	5(B)	4(A)	4(C)	4(C)	4(C)
Crystalline B Vitamins	6× weekly orally	6× weekly orally	Per 10 g. Diet [5]	Per 10 g. Diet [5]	Per 10 g. Diet [6]	Per 10 g. Diet [6]	Per 10 g. Diet [6]
Thiamine HCl γ	30	30	20	20	50	50	50
Pyridoxine HCl γ	20	20	20	20	50	50	50
Riboflavin γ	40	40	50	50	100	100	100
PABA γ			50	50	100	100	100
Niacin γ			100	100	200	200	200
Calcium Pantothenate γ			280	280	500	500	500
Inositol mg.			2	2	4	4	4
Choline HCl mg.	1	1	5	5	10	10	10

[1] 1 cc. of the fat-soluble vitamin mixture furnishes 650 mg. corn oil (Mazola), 800 U.S.P. Units vitamin A, 116 chick units vitamin D and 6 mg.  $\alpha$ -tocopherol (Merk).

[2] Salt mixture A is salts No. 185 of McCollum and Simmonds(4); Salt mixture B is a modification of salts No. 1 of Jukes (5); Salt mixture C is salts No. 4 of Hogsted *et al.* (6).

[3] 0.5 mg.  $\alpha$ -tocopherol (Merk) is given 6× weekly to each rat; the cod liver oil present in the diet furnishes vitamins A and D.

[4] Acid-washed casein is used in this diet; all other diets contain alcohol-extracted casein.

[5] The vitamins were incorporated in the diet as follows: inositol, a sucrose-riboflavin mixture, and a 20% alcoholic solution containing the remainder of the vitamins.

[6] A dry mixture of sucrose with all the crystalline vitamins except choline was used. The choline was kept in a vacuum desiccator and the required amount quickly weighed out whenever needed.

to weight. All rats were maintained in individual cages provided with screens to prevent coprophagy. The day before parturition was expected to occur and throughout the 21-day lactation period, shavings were used instead of screens. Female rats were bred at 3 months of age and usually rebred twice; vaginal smears were examined daily. Male rats were bred at 90-100 days of age with normal females and every 7-14 days thereafter.

The composition of the purified diets used is shown in Table I. Littermate controls were maintained on the stock Diet I<sup>1</sup> plus lettuce *ad libitum* for normal controls.

The purified diets used fall into 3 groups: *Series A*: Control diets used for "Filtrate Factor" studies; *Series B*: Diets varying in B vitamin supplements, casein treatment, and salt mixtures; and *Series C*: Improved basal diets high in carbohydrate, protein or fat. These diets contained higher levels of the B vitamins than used previously and a better salt mixture. Each series required 10-12 months to complete.

Tables II and III summarize the data on reproduction and lactation. The criteria used for reproduction in male rats are: (1) percentage of positive matings, and (2) percentage of the normal mated females showing implantation. The criteria used for reproduction in female rats are: (1) number of young per litter or average size of litter, (2) percentage of young born dead and (3) average weight of the young at birth. The criteria for lactation are: (1) percentage of young weaned, (2) average weaning weight of the young and later (3) the weight change of the lactating mother.

### *Series A: Filtrate Factor Control Diets*

This series revealed the marked inadequacies of the basal diet supplemented with 5 crystalline B vitamins or even with crude sources of the vitamin B complex such as rice bran extract or Northwestern yeast. In agreement with other investigators, lactation was found to be the function most adversely affected. Growth was markedly subnormal. The differences between the stock and purified diets were accentuated in the second generation, where additional abnormalities appeared.

Typical growth curves for female rats in this series are given in Fig. 1. The growth of rats receiving no supplements save the 5 crystalline vitamins (Diet 820 plus CaP) was subnormal in the first generation and greatly decreased in the second generation (Curves A). The substitution of a rice bran concentrate for pantothenic acid improved growth to some extent in both generations (Curves B). Supplementation with Northwestern yeast resulted in normal growth in the first

<sup>1</sup> This is a slight modification of McCollum's Diet I containing 67.5% ground whole wheat, 15% technical casein, 10% whole milk powder, 5.25% hydrogenated vegetable oil (Crisco), 1.5% calcium carbonate and 0.75% sodium chloride. 3.5 grams Sardilene (fish oil concentrate containing 3,000 U.S.P. vitamin A and 400 Chick Units vitamin D/g.) are added to each kg. of diet. The approximate composition is 24.3% protein, 54.8% carbohydrate and 11.0% fat.



TABLE II  
*Breeding Record of Male Rats Maintained on Purified Diets  
 and on a Natural Food Stock Diet*

Exptl Diet	Number of Rats Bred <sup>b</sup>	Number of Oestrus Females Offered	"Refused" Mating Per cent	Matings Plug & Sperm Per cent	Mated Females Showing Implantation Per cent
Series A					
820+CaP <sup>1</sup>	G <sub>1</sub> 3	30	3	97	100
	G <sub>2</sub> 2	10	100	0	
820+Rice Bran Extract <sup>2</sup>	G <sub>1</sub> 3	30	3	97	100
	G <sub>2</sub> 7	34	26	74	96
820+Yeast <sup>3</sup>	G <sub>2</sub> 7	39	10	90	100
Series B					
820+CaP <sup>4</sup>	G <sub>2</sub> 1	14	11	86	92
820+CaP, <sup>5</sup> PABA, Inositol	G <sub>2</sub> 6	19	37	63	75
830	G <sub>2</sub> 2	6	33	67	100
831	G <sub>2</sub> 7	24	29	71	71
Series C					
836 (High CHO) From Birth	G <sub>1</sub> 8	37	19	81	97
837 (High Protein) From Birth	G <sub>1</sub> 7	35	14	86	100
838 (High Fat) From Birth	G <sub>1</sub> 7	35	9	91	97
Stock Rats					
Stock Diet 1 +lettuce <i>ad libitum</i>	G <sub>1</sub> and 28 G <sub>2</sub>	126	9	91	97

<sup>1</sup> 150  $\gamma$  calcium pantothenate given orally 6 $\times$  weekly.

<sup>2</sup> 1 cc. Galen B given orally 6 $\times$  weekly.

<sup>3</sup> 1 g. Northwestern yeast given orally 6 $\times$  weekly.

<sup>4</sup> 200  $\gamma$  calcium pantothenate given orally 6 $\times$  weekly.

<sup>5</sup> 200  $\gamma$  calcium pantothenate, 1 mg. *p*-aminobenzoic acid (PABA) and 2 mg. inositol given orally 6 $\times$  weekly.

<sup>6</sup> G<sub>1</sub>—first generation animals; G<sub>2</sub>—second generation animals maintained on the same diet.

TABLE III

*Reproduction and Lactation of Female Rats Maintained on Purified Diets and on a Natural Food Stock Diet*

Expt Diet	No Rats Bred <sup>a</sup>	No of Litters	Total No Young	Young Born Dead Per cent	Av Size of Litter	Av Wt Day 1	Av Wt Day 21	Weaned Day 21 Per cent
Series A								
820+CaP <sup>1</sup>	G <sub>1</sub> 7	21	135	16	6.4	5.0	22 <sup>1</sup>	14
	G <sub>2</sub> 1	0					19	
820+Rice Bran Extract <sup>2</sup>	G <sub>1</sub> 2	6	50	4	8.3	5.3	33 <sup>+</sup> 29	58
	G <sub>2</sub> 5	8	55	9	6.9	5.0	22 <sup>+</sup> 24	11
820+Yeast <sup>3</sup>	G <sub>1</sub> 4	11	99	9	9.0	5.4	34 33	21
	G <sub>2</sub> 6	7	36	0	5.1	5.3	35 33	21
Series B								
820+CaP <sup>1</sup>	G <sub>1</sub> 5	5	37	8	7.4	1.8	25 26	40
	G <sub>2</sub> 3	2	12	42	6.0	5.0	—	0
820+CaP, PABA, Inositol <sup>5</sup>	G <sub>1</sub> 6	6	54	2	9.0	4.9	25 23	31
	G <sub>2</sub> 3	2	17	0	8.5	4.7	—	0
822+CaP <sup>4</sup>	G <sub>1</sub> 5	5	43	7	8.6	5.0	17	67 <sup>7</sup>
	G <sub>2</sub> 0						15	
830	G <sub>1</sub> 5	6	52	2	8.7	5.4	29 27	77
	G <sub>2</sub> 16	13	83	2	6.4	5.3	28 25	57
831	G <sub>1</sub> 6	6	67	6	11.2	4.3	20 23	20
	G <sub>2</sub> 5	5	25	12	5.0	4.3	—	0

TABLE III—*Continued*

Expt Diet	No Rats Bred <sup>1</sup>	No. of Litters	Total No Young	Young Born Dead Percent	Average Size of Litter	Average Wt Day 1	Average Wt Day 21	Weaned Day 21 Percent
Series C								
836 (High CHO) From Birth	G <sub>1</sub> 5	14	127	2	9.1	5.8	<sup>♂</sup> 37 35	90
837 (High Protein) From Birth	G <sub>1</sub> 7	20	153	0	7.7	6.4	<sup>♂</sup> 40 38	89
838 (High Fat) From Birth	G <sub>1</sub> 7	18	160	1	8.9	5.3	<sup>♂</sup> 33 28	56
Stock Rats								
Stock Diet I+lettuce <i>ad libitum</i>	G <sub>1</sub> and G <sub>2</sub> 26	40	406	1	10.1	5.8	19 18	93
Average for Colony (1938)	100	100	880	2	8.8	6.0	Same	89-93

<sup>1</sup> 150-200  $\gamma$  calcium pantothenate given orally 6 $\times$  weekly.

<sup>2</sup> 1 cc. Galen B given orally 6 $\times$  weekly.

<sup>3</sup> 1 g. Northwestern Yeast given orally 6 $\times$  weekly.

<sup>4</sup> 200  $\gamma$  calcium pantothenate given orally 6 $\times$  weekly.

<sup>5</sup> 200  $\gamma$  calcium pantothenate, 1 mg. *p*-aminobenzoic acid and 2 mg. inositol given orally 6 $\times$  weekly.

<sup>6</sup> G<sub>1</sub>—first generation animals; G<sub>2</sub>—second generation animals maintained on the same diet.

<sup>7</sup> None of these young survived to thirty days of age.

<sup>1</sup> In this column the upper figure represents the average body weights of male rats and the lower figure the average body weights of female rats.

generation but subnormal in the second (Curves C). The differences in growth between groups were the same for male as for female rats but the greater growth potential of the male accentuated the differences.

Reproductive behavior by male rats (Table II) was excellent in the first generation for all groups but impaired in the second generation as judged by the percentage of matings for the groups receiving calcium pantothenate or rice bran extract.

Reproduction in female rats (Table III) receiving no supplements save the 5 crystalline vitamins was subnormal in the first generation and a complete failure in the second generation. Supplementation with rice bran or yeast improved reproduction in both generations but was more effective in the first generation.

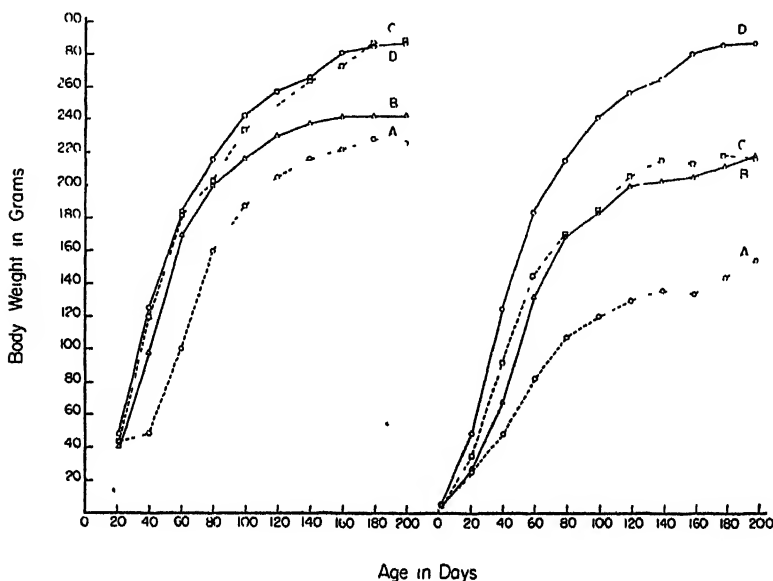


FIG. 1. Typical Growth Curves for Female Rats in Series A.

Left Graph - First generation animals maintained on (A) Diet 820 plus 150  $\gamma$  calcium pantothenate 6X weekly; (B) Diet 820 plus 1 cc. rice bran extract (Galen B) 6X weekly; (C) Diet 820 plus 1 g. Northwestern Yeast 6X weekly; (D) Stock Diet 1 plus lettuce *ad libitum*. (6 rats/dietary group.)

Right Graph - Second generation animals maintained on the identical diets. (A) 5 rats; (B) 8 rats; (C) 6 rats; (D) 5 rats.

Striking differences between the stock and the purified diets were revealed during lactation (Table III). Only 14% of the 21 litters maintained on the basal diet supplemented with the 5 crystalline vitamins were weaned and the weaning weights of the young were markedly subnormal. Supplementation with rice bran extract or with yeast improved lactation to some extent, with the rice bran group being better in the first generation and the yeast group better in the second.

There were no differences in the general condition of the animals during the first generation but in the second generation the animals receiving only crystalline vitamins as supplements showed marked abnormalities. Shortened extremities (confirmed by X-ray studies), apparent exophthalmos,<sup>2</sup> dermatitis either localized or extending over the entire body, and greasy, greyish fur were exhibited by practically all animals. Histological examination of tissues revealed some thyroid hyperplasia, but all other organs were normal in appearance or showed only the changes due to inanition. Animals receiving rice bran extract exhibited none of the above abnormalities. Rats receiving yeast showed them in a milder form which was later in appearance.

In this series, supplementation with two crude sources of the vitamin B complex resulted in some improvement, showing that additional known or unknown B vitamins should be added to the purified diet. However, the fact that such supplementation did not result in normal growth, reproduction and lactation during the two generations indicated that other changes were necessary in the diet, *e.g.*, improvements in other constituents such as the protein or fat components or the salt mixture, or better sources of the vitamin B complex.

*Series B: Diets Varying in B Vitamin Supplements,  
Casein Treatment and Salt Mixtures*

In this series inositol, *p*-aminobenzoic acid and niacin were added to the B vitamin supplements, the choline level was increased and the B vitamin mixture was fed orally or incorporated in the diet.<sup>3</sup> Salt mixture B was substituted for salt mixture A because the former contains the trace elements found to be important in nutrition since the McCollum salt mixture (salts A) was first formulated. In one diet, acid-washed casein was employed in place of the usual alcohol-extracted casein.

The addition of inositol and *p*-aminobenzoic acid did not improve growth (Fig. 2), reproduction in males (Table II) or lactation in fe-

<sup>2</sup> Martin (7) reported that Rockland strain black rats receiving a purified diet plus 8 crystalline B vitamins developed an apparent exophthalmos after prolonged periods on the diet, 6-12 months, but that the thyroids were atrophied, not hypertrophied.

<sup>3</sup> Incorporation of the vitamins in the diet results in an increased vitamin intake as the food intake increases and should take care of the higher vitamin requirements during reproduction and lactation.

males (Table III). Reproduction in the female was somewhat improved as shown by the greater number of young per litter and the decreased percentage of young born dead. This is confirmed by the improvement in reproduction shown by diet 831 which contains the same B vitamins

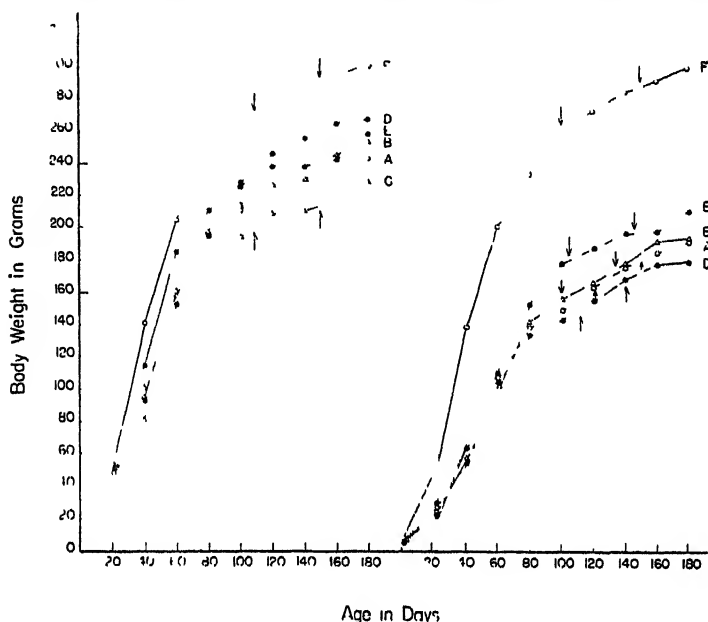


FIG. 2. Typical Growth Curves for Female Rats in Series B. The Arrows Represent the Beginning and End of Gestation.

Left Graph—First generation animals maintained on (A) Diet 820 plus 200  $\gamma$  calcium pantothenate 6 $\times$  weekly; (B) Diet 820 plus 200  $\gamma$  calcium pantothenate, 1 mg. *p*-aminobenzoic acid and 2 mg. inositol 6 $\times$  weekly; (C) Diet 822 plus 200  $\gamma$  calcium pantothenate 6 $\times$  weekly; (D) Diet 830; (E) Diet 831; (F) Stock Diet I plus lettuce *ad libitum*. (6 rats/dietary group).

Right Graph—Second generation animals maintained on the identical diets. (A) 3 rats, (B) 3 rats; (C) curve omitted; rats did not survive 30 days of age; (D) 5 rats; (E) 14 rats, (F) 9 rats.

incorporated in the diet. The presence of niacin in the latter diet, together with an increased level of choline, did not result in any further improvement in reproduction. No improvements in growth or lactation were noted as a result of these two changes.

Reproduction in second generation male rats was much better than in previous tests in Series A (on the few animals receiving no supple-

ments save the crystalline B vitamins). No improvement in reproductive performance over the basal group (Diet 820 plus CaP) was observed in the other experimental groups.

Growth was improved only slightly, if at all, by any of the changes made in the purified diets. The difference between growth on the stock diet (Curves F) and that on the various purified diets during the first generation is again accentuated in the second generation.

The use of salt mixture B together with the acid-washed casein in diet 830 resulted in marked improvement in lactation for both generations. The percentage of young weaned and their average weight at weaning are the highest of any dietary groups in either the first or second series. Growth of second generation animals in this group appeared to be slightly better than in the other dietary groups. This is probably a reflection of the improvement shown in lactation, *i.e.*, in the weaning weights of the young.

Diet 822,<sup>4</sup> which contained salt mixture B together with alcohol-extracted casein, showed a temporary improvement in lactation, *i.e.*, the percentage of young weaned was high (67%) but the young did not survive 30 days of age. Growth of the first generation animals was lower than in the other dietary groups.

In this series the addition of inositol and *p*-aminobenzoic acid improved reproduction in female rats in the first generation. The addition of niacin and of increased choline did not lead to further improvement in reproduction. Lactation in both generations was strikingly improved when salt mixture B together with an acid-washed casein was used.

#### *Series C: Improved Basal Diets High in Carbohydrate, Protein, or Fat*

Salt mixture C<sup>5</sup> was used in this series because of the difficulties encountered with salt mixture B.<sup>1</sup> Higher levels of the B vitamins were incorporated as a dry mixture in the diet, as previous attempts

<sup>4</sup> This diet had poor keeping qualities as did diet 830 which contained the same salt mixture. Using fresh diet every day did not eliminate the development of odors and marked discoloration of the diets. This instability was more marked when alcohol-extracted casein was used than when acid-washed casein was used. Because of the instability of these diets, it was difficult to tell whether the change in the salt mixture or in the casein treatment was responsible for the improvement in lactation, although it was thought that the former change was more important.

<sup>5</sup> Salt mixture C contains the same trace elements but in different proportions than in salt mixture B. No instability of the diets containing salt mixture C has been observed.

to use a more concentrated vitamin solution resulted in diets of poor keeping qualities.<sup>6</sup> Since the basal diet in all previous experiments had been high in carbohydrate, high protein and high fat diets with the same salt and vitamin mixtures were also tested at the same time.

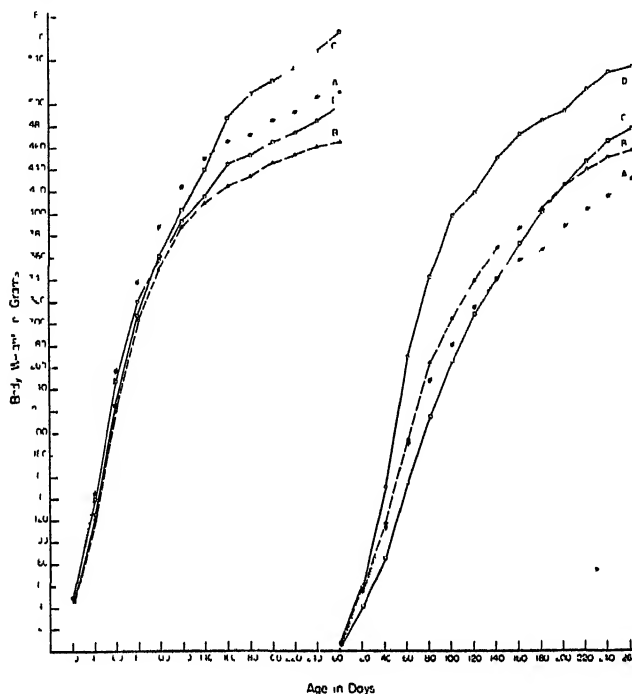


FIG. 3. Typical Growth Curves for Male Rats in Series C.

Left Graph—Growth of first generation animals (weanlings) on (A) High carbohydrate Diet 836; (B) High protein Diet 837; (C) High fat Diet 838; (D) Stock Diet I plus lettuce *ad libitum*. (10 rats/dietary group.)

Right Graph—Growth of first generation animals placed on the identical diets at birth, 21 days earlier. (7 rats/dietary group.)

*High Carbohydrate Diet (836).* Fig. 3 shows the striking improvement in growth of weanling rats<sup>7</sup> during the first generation. Growth on the high-carbohydrate diet (Curve A) was consistently equal and even slightly superior to that on the stock diet (Curve D). This slight superiority in growth on diet 836 in comparison to the stock diet has

<sup>6</sup> Instability of the choline was suspected.

<sup>7</sup> Rats placed on the experimental diets at weaning, the usual procedure.



been noted in every series of litter mates studied during the past 2 years. On the other hand, when the diets are started at birth, the usual decrease in growth found in second generation rats maintained on purified diets, is shown in the first generation. Starting the experimental diets at birth includes the crucial period of lactation and reveals the differences in diets much more quickly than does the standard procedure of starting at weaning, 21 days later.

Reproduction and lactation were then studied in these rats placed on the experimental diets at birth, a procedure much more severe and sensitive than ordinarily used for animals of the first generation. However, reproduction tests in male rats (which were more extensive than in the previous 2 series) gave results closely approaching normal values. The percentage of matings was slightly lower than normal and probably reflected the differences in the growth curves. Reproduction in female rats was normal, the weight of the young at birth being higher than in any previous purified diet group. This high standard of reproductive behavior in both male and female rats was obtained despite the severity of the experimental procedure.

The improvement shown in lactation is striking. For the first time the percentage of young weaned by animals maintained on a purified diet reached 90%, which is equivalent to that obtained from stock rats. Furthermore, the weaning weights of the young are higher than any obtained previously with purified diets, although the young are still not equal in weight to stock rats.

*High Protein Diet (837).* On the 64% casein purified diet, growth, reproduction and lactation were very similar to that observed on the high carbohydrate diet. When the rats were placed on the diets at weaning, growth started more slowly and remained somewhat inferior to that on the high-carbohydrate diet (and the stock diet also) throughout the experiment. When the rats were placed on the diets at birth, the high-protein diet was slightly better for growth. There were no significant differences between the two diets in regard to reproduction in either male or female rats. Lactation on the high-protein diet was slightly better since the weaning weights of the young were higher.

*High Fat Diet (838).* On this diet growth started slowly but finally surpassed that of the other purified diet groups. This slow initial growth was more noticeable when the diet was started the day of birth rather than the day of weaning. Lactation on the high-fat diet was obviously inferior to that on the other purified diets. The percentage of young

weaned was markedly decreased and the weaning weights of the young were lower. This result was not expected in view of the normal reproductive behavior in both males and females on this diet and the slight differences in growth in comparison with the other purified diets. Working with lower levels of fat Evans *et al.* (8) and Maynard and Rasmussen (9) showed that lactation improved when the fat content of the diet increased. Vinson and Cerecedo (1) found that a lard supplement, given *ad libitum*, was injurious to lactation performance and suggested that the consumption of such large amounts of lard (the rats showed an abnormal appetite for the supplement) interfered with the ingestion of other food constituents. The fat employed here, hydrogenated cottonseed oil, was incorporated in the diet, not given *ad libitum*, and the food intake was decreased in comparison to that of the high-carbohydrate and high-protein purified diets. Further work will be necessary to establish the exact mechanism of the deleterious effect of high fat on lactation.

*Weight Change of Lactating Rats.* In agreement with Vinson and Cerecedo (1, 2)<sup>8</sup> a marked loss in weight in the majority of the lactating mothers maintained on all purified diets was observed. This weight loss was not invariable as occasionally a mother on the high-carbohydrate or high-protein diet would gain weight. However, the average weight change<sup>9</sup> for any purified dietary group correlated with the other criteria for lactation, *i.e.*, the average weight of the young at weaning and the percentage of young weaned. Rats on the high-carbohydrate diet lost an average of 31 g. (+22 to -60) while those on the high-protein diet, in which the weaning weights of the young were higher, averaged a 21 g. loss (+10 to -44). The marked inferiority of the high-fat diet for lactation was confirmed by an average weight loss of 78 g. (-59 to -114). Rats on the stock diet averaged a gain of 25 g. (0 to +62). This weight change proved to be very useful in short-term studies of lactation (11).

We may say, in summary, that in this series the use of salt mixture C and higher levels of the B vitamins resulted in greatly improved growth, reproduction and lactation. Growth in animals started the day of birth was not as satisfactory as that of animals started at weaning and reflected the strain of lactation. The weight loss of lactating mothers on purified diets was confirmed and found to correlate with the other criteria for impaired lactation, *i.e.*, decreased percentage of young weaned and decreased weaning weights. Increasing the pro-

<sup>8</sup> Morse and Schmidt (10) have also reported a loss in weight during lactation on purified diets.

<sup>9</sup> Only those mothers weaning 5-6 young are included in these averages as the weaning of less young is not as great a strain on the mother.

tein level in the diet resulted in slightly better lactation while increasing the level of fat exerted a markedly deleterious effect on lactation.

### DISCUSSION

In these studies on growth, reproduction and lactation, the primary purpose has been that of immediately improving performance without investigating the complicity of single factors. Frequently, 2 or more factors in the diet were changed at the same time so that responsibility for the improvement shown was difficult to fix. It is obvious and to be expected that the substitution of modern salt mixtures for McCollum's salts (salts A) would give better results in highly purified diets. The use of distilled water instead of tap water for drinking purposes probably increased the importance of the salt mixture.

While excellent growth and reproduction were obtained in Series C where 8 crystalline B vitamins were used, this does not prove that all 8 vitamins, especially the last 3 added (inositol, *p*-aminobenzoic acid and niacin) are needed by the rat during generation studies. The data for Series B indicate that the addition of inositol and *p*-aminobenzoic acid together improved reproduction in female rats and that the further addition of niacin gave no further improvement. The dispensability of dietary niacin for reproduction in the rat has been reported (12). The beneficial effects of inositol and *p*-aminobenzoic acid on reproduction are in agreement with the findings of Sure (13), although Vinson and Cerecedo (1) did not employ either of these 2 vitamins or niacin in their generation studies. However, the latter investigators used 30% casein<sup>10</sup> in their diets in contrast to the 18–20% levels used by the majority of workers. Sure (13), Olimenko and McChesney (15) and Richardson and Hogan (3) have all reported the beneficial effects of inositol and/or *p*-aminobenzoic acid for lactation. The failure of these two vitamins to improve lactation in Series B was probably due to the greater effects of other deficiencies in the diet (particularly deficiencies in the salt mixture).

While the improved basal diets used in Series C result in better lactation than has been reported by other investigators using only crystalline vitamins, some factor(s) is still needed for optimal lactation. Some further studies of the factors affecting lactation in short-term experiments are reported elsewhere (11). It may be mentioned

<sup>10</sup> Purified caseins contain varying amounts of known and unknown B vitamins (14).

that it is difficult to compare lactation results from different laboratories, either on the basis of percentage of young weaned or on the average weaning weight of the young, unless litters are limited to a constant number of young. This point has been emphasized by many investigators of lactation, *e.g.*, Daggs (16). In our stock colony, the litters are limited to 6 young at birth and 90% of the young are weaned with average body weights of 48-50 g. In stock colonies where the litters are unlimited and, therefore, larger in size, the weaning weights of the young will be markedly decreased and the percentage of young weaned will probably be lower.<sup>11</sup> For example, Vinson and Cerecedo (1) reported that stock rats of the Long-Evans strain in their colony weaned litters of 9-10 young with average body weights of 25 g. From the data given approximately 80% of the young were weaned. Such a decrease in weaning weights will naturally affect growth following weaning.<sup>12</sup> Inspection of the growth curves of Vinson and Cerecedo shows that their stock male rats at 85-90 days of age averaged 240 g. (range 205-275) for different groups whereas stock male rats of the same age and strain in our colony average 350 g. in body weight.

#### SUMMARY

Long-term studies have been made of growth, reproduction and lactation in the rat maintained on various purified diets. Lactation was the function most adversely affected by inadequacies in these purified diets. Growth in either male or female rats was the next most sensitive criterion while reproductive behavior in males was the least sensitive.

As a result of these studies an improved basal diet was formulated; it contained higher levels of the 8 crystalline B vitamins and an improved salt mixture. On this diet growth of weanling rats was slightly superior to that produced by stock diets and reproduction closely approached normal values. Growth in animals started the day of birth was not as satisfactory as that of animals started at weaning.

<sup>11</sup> The stock diet used in the colony is also an important factor. Purina Dog Chow, which is frequently used as stock diet, has never resulted in optimum growth, reproduction and lactation in our stock colony.

<sup>12</sup> The use of such small weanling rats would also be expected to affect the severity of dietary deficiencies and the incidence of symptoms, *etc.*, just as the use of a different strain of rat does in many cases.

Lactation on the new basal diet was greatly improved, especially in regard to the percentage of young weaned. This percentage was equivalent to that of stock rats but growth of the young during lactation, while at a high level, was still not optimal. The loss in weight of the lactating mothers maintained on all purified diets in contrast to the gain in weight of stock rats persisted and is thus to be added to the other evidence for defective lactation. Increasing the dietary protein level resulted in slightly better lactation while increasing the level of fat exerted a markedly deleterious effect.

The requirements for growth in weanling rats and for reproduction are satisfied by the new basal diet but the function of lactation has additional dietary requirements.

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## Lactation on Purified Diets<sup>1</sup>

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Long-term studies by Nelson and Evans (1) of growth, reproduction and lactation in the rat maintained on purified diets resulted in the formulation of an improved basal diet on which growth and reproduction in both sexes were normal but lactation, although greatly improved, was not yet optimal. Since the strain of lactation in the mother was reflected immediately in the decreased growth of suckling young, the mothers of which were placed on purified diets at birth, it was thought that a short-term procedure might be used to advantage in determining the unknown factor(s) necessary for optimal lactation. The simple procedure of placing adult stock animals with their litters on the experimental diets at parturition makes it possible to test many dietary factors for their effects on lactation in 3 weeks, whereas long-term studies of lactation require a minimum of 8-10 months. This report will show that the effects of the unknown deficiencies in the purified diets are almost as severe in the short-term procedure as in the longer studies and that the short procedure can be used in the search for the unknown dietary factors required in lactation by the rat.

### EXPERIMENTAL PROCEDURE

Rats of the Long-Evans strain were used in all experiments. Adult stock animals, 4-7 months of age, together with their litters, were placed on the purified diets at parturition. The litters were limited to 6 young, preferably 3 males and 3 females.

<sup>1</sup> Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: crystalline B vitamins from Hoffman-La Roche Company, Nutley, New Jersey, and Merck and Company, Inc., Rahway, New Jersey; alpha-tocopherol from Merck and Company, Inc., Rahway, New Jersey; 93% alcohol liver concentrate (II-9381-B) and liver fraction powder (Lab. No. 7-5293) from Lederle Laboratories, Inc., Pearl River, New York.

<sup>2</sup> General Mills Fellow.

All animals, mother and young, were weighed every 5 days and the young were weaned on the 21st day following parturition.

A minimum of 10-12 litters were used for each experiment, 3 or 4 litters being started each week until the series was completed. Three or 4 different experimental groups, together with stock control litters, were run simultaneously to minimize variations in lactation performance for different weeks. For the same reason, an additional series of 10-12 litters was repeated 2-6 months later for the majority of experiments.

The criteria used for lactation efficiency with this procedure are: (1) the average weight of the young at weaning and (2) the weight change of lactating mothers during the 21-day period. Only those mothers weaning 5-6 young were included in the average weight change as the weaning of less young is not as great a strain on the mother.\* Since the percentage of young weaned on purified diets (high in carbohydrate or protein) during long-term studies was equivalent to that of stock rats in our colony (*i.e.*, 90%) this criterion has been eliminated from our work.

The purified diets used are the improved basal diets previously reported (1), *i.e.*, the high-carbohydrate diet (836),<sup>4</sup> the high-protein diet (64% casein) and the high fat diet (48% fat). Any changes in the basal high-carbohydrate diet were made at the expense of the sucrose. The stock Diet I (1) plus lettuce *ad libitum* was used for control litters.

## RESULTS AND DISCUSSION

### *Comparison of Short-Term Procedure with Long-Term Studies*

Table I compares the data obtained by the short-term procedure with that obtained by long-term studies on identical diets.<sup>5</sup> Both

<sup>3</sup> When 2-4 young are weaned, the mother on a purified diet usually gains weight during lactation. There was no significant difference in the weight change of mothers weaning 5 young and those weaning 6 young on the same diet, *i.e.*, the variation between individual mothers was greater than the difference in the strain of weaning 5 or weaning 6 young.

<sup>4</sup> The basal high-carbohydrate diet (836) consists of 24% alcohol-extracted casin, 64% sucrose, 8% hydrogenated vegetable oil (Crisco) and 4% salts (2). Crystalline B vitamins are added per kg. diet: thiamine HCl 5 mg., pyridoxine HCl 5 mg., riboflavin 10 mg., *p*-aminobenzoic acid 10 mg., nicotinic acid 20 mg., calcium pantothenate 50 mg., inositol 400 mg., and choline chloride 1 g. One cc. of a fat-soluble vitamin mixture containing 6 mg.  $\alpha$ -tocopherol, 115 Chick Units vitamin D, 800 U.S.P. units vitamin A and 350 mg. corn oil (Mazola) is given weekly to each litter.

<sup>5</sup> Long term or generation studies are based on the performance of animals maintained for one or more generations on the same diet. The production of 3 litters each by first generation animals requires a minimum of 8-10 months. The data given in the table for comparison with that obtained by the short-term procedure are from first generation animals maintained on the purified diets from birth, a more severe test than the usual procedure in which animals are started at weaning, 21 days later (1).

TABLE I

*Comparison of Long-Term and Short-Term Studies of Lactation on Purified Diets*

Experimental diet	No. of litters	No. of young	Av. wt. day 1	Av. wt. day 21	Weaned day 21	Weight change of mother during lactation <sup>1</sup>
<i>a</i> <i>a.</i> per cent <i>a.</i> <i>First Generation Animals Maintained on Purified Diets (Long-Term Studies)</i>						
High CHO	14	42♂ 42♀	6.1 5.9	37 35	90	-31 g. (13) (+22 to -64)
High protein	20	58♂ 59♀	6.3 6.0	40 38	89	-21 g. (17) (+10 to -44)
High fat	18	49♂ 55♀	6.0 5.7	33 28	56	-78 g. (4) (-59 to -114)

*Stock Animals Placed on Purified Diets at Parturition (Short-Term Studies)*

High CHO	20	60♂ 59♀	6.2 5.9	41 43	93	-25 g. (18) (-5 to -54)
High protein	20	63♂ 56♀	6.4 6.0	44 41	93	-21 g. (20) (-1 to -52)
High fat	24	75♂ 69♀	6.3 5.9	35 34	96	-63 g. (23) (-11 to -90)

*Stock Animals Maintained on Stock Diet I + Lettuce ad libitum*

Stock High CHO	25	74♂ 76♀	6.3 5.9	50 48	96	+23 g. (21) (+62 to -5)
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<sup>1</sup> The number of animals on which the average weight change is based is given in parentheses (superscript number). The range is given in parentheses below the average.

procedures reveal the inferiority of the purified diets to the stock diet for lactation. Lactation performance is somewhat better in the short-term procedure than in the longer procedure, as is shown by the improved weaning weights of the young for all purified diets. In addition,



the weight loss of mothers given the high-carbohydrate diet is somewhat decreased.<sup>6</sup>

Storage of the unknown factor(s) required during lactation is shown by the improvement in performance during the short-term procedure but that such storage is limited is demonstrated by the superiority of the stock diet. Variability in storage or in requirement is shown by the range given for the weight change of the lactating mother. Such variation is to be expected if age, body weight and/or intestinal synthesis are concerned in the requirement for the factor.

It may be noted that the slight superiority of the high-protein diet in comparison with the high-carbohydrate diet as shown in long-term studies is lost in the short procedure. This may be due to the fact that the high-protein diet is the only purified diet used in which a short period of adjustment to the diet would be beneficial. On all other purified diets the rats gain in weight during the first 5 days of lactation and seem to experience no difficulty in shifting from the natural food stock diet to the more concentrated purified diets. Another possibility is that the high-protein level may exert its beneficial effects in the longer studies during a different period, *e.g.*, gestation when the rat stores additional protein if possible (3). The protein level used (64% casein) is probably far beyond the optimum level<sup>7</sup> needed for lactation, but the results show that the purified casein does not contain significant amounts of the unknown factor(s) necessary for lactation (unless one assumes that the requirement increases directly with the protein level).

The deleterious effect of high-fat diets on lactation is strikingly shown in the short procedure. In fact, the impairment in lactation is almost as great in 3 weeks as in the previous long-term studies when the animals were maintained on the same diet for 8-10 months. The percentage of young weaned is the only criterion to show marked improvement in the short study.

<sup>6</sup> The slightly higher percentage of young weaned in the short procedure for the high-carbohydrate and high-protein diets is insignificant because any failure of lactation during the first 5 days (which occurs occasionally with stock rats, especially with first litters) is not included in the summary of data for the short procedure.

<sup>7</sup> Studies on the optimum protein level for both reproduction and lactation on purified diets are in progress.

*Comparison with Non-Lactating Controls*

To show that the strain of lactation alone is responsible for the differences between the purified diets and the stock diet during the 3-week test period, non-lactating control animals (mothers) were studied. The high-carbohydrate purified diet (836) and the stock diet I plus lettuce, also high in carbohydrate, were used. Litters were paired according to the age and body weight of the mother. In the non-lactating group all litters were weighed at parturition and were then discarded. The food intake of the mother or of the mother plus litter was determined every 5 days.

TABLE II

*Comparison of Lactating with Non-Lactating Animals on a Purified Diet and on a Natural Food Diet (Short-Term Studies)*

Experimental diet	No. of litters	No. of young	Av. wt. day 1	Av. wt. day 21	Weaned day 21	Wt. change of mother during period	Av daily food intake	Body wt at parturition	Age at parturition
			g.	g	per cent	g.	g.	g.	days
<i>Lactating Rats with Litters</i>									
Stock diet I + lettuce	12	37 ♂ 35 ♀	6.3 6.2	52 50	100	+ 14.2 g. (0 to +28)	37.1 (29.9-40.9)	269	198
Purified diet 836	12	38 ♂ 34 ♀	6.4 6.1	43 41	94	- 20.9 g. (-52 to -6)	27.7 (20.0-31.4)	276	187
<i>Non-Lactating Controls</i>									
Stock diet I + lettuce	12	39 ♂ 33 ♀	6.4 6.0	- -	-	+ 4.4 g. (-14 to +33)	14.0 (11.0-19.6)	269	200
Purified diet 836	12	33 ♂ 39 ♀	6.2 5.8	- -	-	+ 8.0 g. (-12 to +27)	14.3 (11.7-17.3)	276	188

Table II shows that, for non-lactating control animals, the purified diet was equivalent and even slightly superior to the stock diet as judged by the average weight gain of the mother. This is in agreement with the optimal growth obtained in weanling rats maintained on this purified diet for several months and emphasizes again the addi-

tional dietary requirements imposed on the animal by the strain of lactation.

It is interesting to note the marked increase in food intake for both diets during lactation and the fact that lactating stock rats gain more weight than non-lactating stock rats during the 21-day period following parturition.

### *Effect of Varying Levels of Fat*

In view of the marked impairment of lactation observed on the 48% fat diet in comparison with the 8% fat diet (either high-carbohydrate or high-protein), it was thought desirable to investigate other dietary levels of fat. Table III shows the inverse relationship between lactation performance and the fat content of purified diets. As the percentage of fat decreases from 48% to 0%, lactation improves. The weight loss of the lactating mother decreases with each decrease in dietary fat while the weaning weights of the young increase. The optimal weaning weights occur at the 8% level; no improvement was observed at the 0% level.

Since the food intake is less on the diets containing higher levels of fat, the vitamin intake was increased to eliminate the possibility of inadequate vitamin levels. Both the crystalline B vitamins in the diet and the amount of the fat-soluble vitamin mixture given weekly were doubled. This resulted in a slight improvement in the weaning weights of the young maintained on the 48% level but there was no significant change in the weight loss of the mother. The defective lactation on this diet is not, therefore, due primarily to decreased vitamin intake. This is confirmed by the lack of beneficial effects at the 8%<sup>8</sup> level when the vitamin intake was doubled. This showed, furthermore, that the improvement in lactation at the 0% level was not due to an increased vitamin intake.

Although the 0% fat diet contains more carbohydrate (72%) than any other purified diet, there is no evidence to indicate that sucrose *per se* benefits lactation. It will be remembered that lactation performance on the high-protein diet containing only 24% sucrose was equivalent to that on the 64% sucrose diet (high-carbohydrate). It seems more logical, therefore, to conclude that the improved lactation at the 0%

<sup>8</sup> Doubling the vitamin intake at this level resulted in no improvement for either the high-carbohydrate or high-protein diets, both of which contain 8% fat. The data given in Table III are for the high-carbohydrate diet.

level is due to the lack of fat and that fat is related to the requirement or utilization of the unknown factor(s) essential for lactation. This improvement in lactation when the diet contains no fat (but is supplemented with the essential unsaturated fatty acids) is contrary to the results obtained by other investigators. Evans, Lepkovsky and Murphy (4), who used *ad libitum* feeding, and Maynard and Rasmussen (5), who used the paired feeding technique, found that lactation improved as the fat content of the diet increased. In both of these studies the

TABLE III

*Lactation on Purified Diets of Varying Fat Content (Short-Term Studies)*

fat in diet <sup>1</sup>	No. of litters	No. of young	av. wt. day 1	av. wt. day 21	Weighted day 21	Weight change of mother during lactation*
per cent			g	g	per cent	g
0 <sup>2</sup>	10	30 ♂ 30 ♀	6.2 5.8	41 39	97	-10 g. (10) (+5 to -24)
8	20	60 ♂ 59 ♀	6.2 5.9	41 43	93	-25 g. (18) (-5 to -54)
24	10	30 ♂ 30 ♀	6.1 5.9	33 33	90	-40 g. (9) (-9 to -55)
48	24	75 ♂ 69 ♀	6.3 5.9	35 34	96	-63 g. (23) (-11 to -90)

*Double Vitamin Intake*

8	19	59 ♀ 55 ♀	6.2 6.1	42 41	98	-20 g. (17) (+19 to -66)
48	20	62 ♂ 58 ♀	6.4 5.9	38 37	95	-58 g. (18) (0 to -85)

<sup>1</sup> The percentage of fat was increased or decreased from the basal high-carbohydrate diet containing 8% fat with corresponding changes in the percentage of sucrose, i.e., 0% fat with 72% sucrose, 8% fat with 64% sucrose, 24% fat with 48% sucrose, and 48% fat with 24% sucrose. All other constituents of the diet remained the same.

<sup>2</sup> One cc. corn oil was given weekly to each litter, in addition to the fat-soluble vitamins, to insure an adequate intake of the essential unsaturated fatty acids.

\* The superscript number in parentheses is the number of animals on which the average weight change is based. The range is given in parentheses below the average.

vitamin B complex was present in natural sources and presumably supplied both the known and unknown factors necessary for lactation.

### *Effect of B Vitamin Concentrates*

The preceding experiments have shown that increasing the level of protein, of fat, of the 8 crystalline B vitamins and of the fat-soluble vitamins (known and unknown present in the given mixture) led to no improvement during the 3-week test period. Furthermore, the addition of crystalline biotin to the 8 B vitamins already present in the diet had no marked effects.<sup>9</sup> Several B vitamin concentrates were then tested to see if they contained the missing factor(s) necessary for optimal lactation. The supplements were given orally at first and later incorporated in the diet at a level calculated to furnish approximately the same amount as the oral supplement. The high-carbohydrate diet (836) was used in these studies as it is closest in approximate composition to the stock Diet I used as a standard.

The beneficial effects of these concentrates on lactation are shown in Table IV. Rice bran extract was slightly beneficial, while yeast resulted in greater improvement. Liver extract<sup>10</sup> given orally was about as beneficial as the rice bran extract. When the liver extract was incorporated in the diet (2% level) considerably greater improvement resulted. This difference was thought to be due to the observed instability of the diluted liver extract when fed orally. Increasing the liver extract from 2 to 3% did not show further improvement. In fact, the weight gain of the lactating mothers in the 3% groups was not as great as those receiving the 2% level.<sup>11</sup> It may be noted that 2% liver extract resulted in better lactation than an equivalent amount of yeast.

<sup>9</sup> When biotin was added, the young were slightly heavier at weaning but the weight loss of the mother was greater. Vinson and Cerecedo (6) have reported that biotin had no beneficial effects on lactation.

<sup>10</sup> 93% alcohol liver concentrate (H-9381-B) from Lederle Laboratories, Inc.

<sup>11</sup> It is possible that part of the weight gain in the 2% groups was due to increased coprophagy. Slight to severe diarrhea occurred in many of the animals receiving the liver extract. Whenever diarrhea occurs, it has been found necessary to clean the cages 6 times weekly to reduce coprophagy instead of 3 times weekly, the usual procedure. This additional cleaning was carried out in the 3% liver extract groups. When animals are maintained on shavings, as in the case with lactating animals and their litters, it is impossible to eliminate coprophagy to the extent that can be done when animals are maintained on screens.

TABLE IV

*Lactation on the High CHO Purified Diet (836) Supplemented with  
Various Vitamin B Concentrates (Short-Term Studies)*

Supplement	No. of litters	No. of young	Av. wt. day 1	Av. wt. day 21	Weaned day 21	Weight change of mother during lactation*
None	20	60 ♂ 59 ♀	<i>g.</i> 6.2 5.9	<i>g</i> 44 43	<i>per cent</i> 93	<i>g.</i> -24.8 g. (18) (-5 to -54)
1 cc. Rice bran extract <sup>1</sup> daily	10	30 ♂ 30 ♀	6.6 6.2	47 46	95	-13.0 g. (10) (+3 to -33)
0.5 g. Yeast <sup>2</sup> daily	9	27 ♂ 27 ♀	6.3 6.2	47 44	93	-7.5 g. (8) (+25 to -38)
2% Yeast <sup>2</sup>	12	33 ♂ 39 ♀	6.5 6.2	49 46	96	-4.9 g. (11) (+21 to -27)
0.5 cc. Liver extract <sup>3</sup> daily	8	24 ♂ 24 ♀	6.2 5.8	48 45	96	-14.4 g. (8) (+4 to -41)
2% Liver extract <sup>3</sup>	12	33 ♂ 39 ♀	6.4 6.1	47 46	92	+14.9 g. (10) (+43 to 0)
3% Liver extract <sup>3</sup>	11	35 ♂ 30 ♀	6.3 6.1	48 47	94	+3.6 g. (10) (+48 to -20)
3% Liver extract <sup>3</sup>	9	27 ♂ 27 ♀	6.5 6.1	48 46	98	+6.4 g. (9) (+16 to -15)
0.5% Liver powder <sup>4</sup>	12	36 ♂ 36 ♀	6.3 6.1	50 48	100	+16.8 g. (12) (+32 to -6)

\* Superscript number in parentheses is the number of animals on which the average weight change is based. The range is given in parentheses below the average.

<sup>1</sup> Galen B.

<sup>2</sup> Northwestern Yeast.

<sup>3</sup> 93% alcohol liver concentrate (H-9381-B) from Lederle Laboratories, Inc.

<sup>4</sup> Liver fraction powder (Lab. No. 7-5293) from Lederle Laboratories, Inc.; each g. contains 550  $\gamma$  *L. casei* factor.

The greatest improvement in lactation is shown by the group receiving a liver eluate powder (0.5% level).<sup>12</sup> Normal or practically normal lactation resulted in this group with the weaning weights of the young being equivalent, for the first time on a purified diet, to those of stock rats. The average weight gain of the lactating mothers is within the normal range, although slightly lower than the weight gain usually produced by the stock diet. Further work will be necessary to make sure that the liver powder contains all the missing factors required by the rat.

The liver eluate powder furnishes a high level of folic acid (550  $\gamma$  *L. casei* factor/g.). Beneficial effects on lactation of considerably smaller quantities of folic acid concentrates have been reported by Cerecedo and Vinson (7) on the basis of a few litters tested. However, Richardson and Hogan (8) found that 5% liver filtrate, containing practically no vitamin B<sub>12</sub>, was just as beneficial to lactation as 1% liver eluate which supplied considerable vitamin B<sub>12</sub>. A preliminary test of low levels of folic acid (concentrate) with this short-term procedure led to no improvement in lactation or in maternal leucocytes (Nelson, van Nouhuys and Evans) (9).

In view of the difficulties in measuring the activity of different forms of the *L. casei* factor, it is useless to speculate on the relation of this factor to lactation unless synthetic forms of the vitamin are used. Such studies are in progress and will be reported later.

### SUMMARY

A short-term procedure of 3 weeks has been used and standardized for testing the efficacy of many dietary factors in improving the lactation performance of rats maintained on purified diets. The deficiencies of the improved basal diets for lactation, previously observed in long-term studies, are almost as severe in the short procedure, indicating a limited storage of the missing factors.

The excellent growth of mothers placed on the same basal diet but deprived of their young, showed that the strain of lactation alone accounted for their weight loss when lactating. These results also, of course, again confirmed the fact that the basal purified diet fulfilled all the dietary requirements for growth of rats past the age of weaning.

The inverse relationship between lactation performance and the fat content of purified diets has been shown for diets varying in fat from 0 to 48%. This impairment in lactation on diets high in fat was not due to decreased vitamin intake.

<sup>12</sup> Liver fraction powder (Lab. No. 7-5293) from Lederle Laboratories, Inc.

Several B vitamin concentrates were found to supply varying amounts of the missing factor(s) for lactation. A liver eluate powder was the best source tested and resulted in normal or practically normal lactation during the 3-week period.

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# Dietary Alteration of Enzyme Activity in Rat Liver \*

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## INTRODUCTION

Samuels and coworkers (1-4) have compared groups of rats fed on high-fat or on high-carbohydrate diets and have shown by tissue and blood analyses that fat feeding has a sparing action on carbohydrate utilization during subsequent fasting. In attempting to explain their results they suggested (4) that the effect "may be accomplished by the building up of one group of enzyme systems in the tissues (*e.g.*, that responsible for the combustion of fat) in preference to other systems." Such an explanation is not unreasonable in view of the existing data on micro-organisms (5-8) as well as on mammals (9). However, there is as yet no direct evidence to show that the concentration of the enzymes which perform the oxidative steps in metabolism can be changed by alterations in the fuel<sup>1</sup> supplied. While no changes would necessarily be anticipated in the enzymes of oxidative pathways common to protein, carbohydrate and fat, it is conceivable that the enzyme which oxidizes octanoic acid to acetoacetic acid (11, 12) might be increased in animals fed a high fat diet, especially in view of the indirect evidence cited (1-4). Since recent work in this laboratory had established some of the precautions needed for the study of the octanoate-oxidizing enzyme (12) we measured the activity of this enzyme system in livers from rats fed high-fat and high-carbohydrate diets. The previously developed succinoxidase assay (13) was applied to these tissues in order to facilitate the interpretation of the octanoate data (12). In addition the succinoxidase assay provides some insight into the ca-

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<sup>1</sup> On the other hand changes in the level of enzyme activity have been correlated with changes in the functional capacity of the tissue (See discussion in (10)).

capacity of the Krebs cycle and represents a control on fluctuations in dry weight, fat content, *etc.*, in the livers. The third substrate employed was citric acid, which is here shown to be oxidizable under conditions similar to those employed for the oxidation of octanoic acid. As components of the Krebs cycle citrate and succinate might be expected to be intermediates for both carbohydrate and fat metabolism, in contrast to octanoate. However, the latter would probably be involved in any conversion of carbohydrate to fat.

The succinoxidase assay is believed to be a measure of the succinic dehydrogenase concentration (13) but it is as yet uncertain what is the limiting component of the octanoate and citrate systems as now constituted. Thus the measurements on these systems were made under various conditions.

### EXPERIMENTAL

The study was limited to the assay of livers from rats which had been on several types of diets for varying periods of time. Group I consisted of rats on the stock ration.<sup>2</sup> Group II was on a high-carbohydrate diet consisting of casein 6, salts 4, glucose hydrate<sup>3</sup> 87, and vitamin B complex<sup>4</sup> 3. Group III received a diet in which the glucose was supplanted by 83% hydrogenated cottonseed oil,<sup>5</sup> and the casein was increased to 10% to compensate for the lower food intake. No adjustment was made in salts or vitamins, since the experiment was of short duration. Group IV consisted of rats from Group I which were decapitated and allowed to lie at room temperature for 5 to 45 minutes before the livers were removed for study. Data on only 2 rats from Group IV are reported, since the results were correlated with the length of time that the livers were anoxic, and the data had to be averaged for each time interval.

Rats weighing 100–175 g. and about 2 months old were shifted from stock rations to the carbohydrate diet for about a week and then shifted to the fat diet. Slight weight losses followed by maintenance of weight were noted. The rats were killed after being on the high-fat diet for periods not exceeding 21 days or on the high-carbohydrate diet for from 12 to 32 days. When data were studied according to the length

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<sup>2</sup> Friskies Dog Biscuits, Carnation Company, Milwaukee, Wisconsin.

<sup>3</sup> "Cerelease," obtained from the Corn Products Refining Company.

<sup>4</sup> "Vitab," a source of the B vitamins obtained from the National Oil Products Company, Harrison, New Jersey.

<sup>5</sup> "Primex," obtained from Proctor and Gamble Company.

of time that the rats were on the diets, there was some indication of minimum values on the high-fat diets at from 9 to 13 days, but the variations were such that no conclusive correlations could be proved, and the data from all rats in each group have been averaged.

The livers were prepared for study by homogenization in saline or water as required (12) and all measurements were based upon the rate of oxygen uptake in a conventional Warburg apparatus. using succinate (13), octanoate (12), or citrate as substrate. The latter substrate was studied using the reaction system employed for octanoate oxidation, since it was shown that ATP stimulates the oxidation of this compound (Table I). The mechanism of the activation is as yet unknown, but the

TABLE I  
*The Activation of Citrate Oxidation by Adenosine Triphosphate*

Group	No. of animals	Additions		Average rate of citrate oxidation $Q_{O_2}$ , uncorr.
		Malonate	ATP	
III	13	+	—	1.6 (0.2–3.3)
		+	+	4.1 (2.2–7.1)
		—	+	6.4 (5.0–7.7)

Each flask contained the washed residue from 100 mg. of wet liver which had been prepared as an isotonic saline homogenate. Reaction mixture based upon octanoate system with sodium ions (12). Results reported as  $Q_{O_2}$  of original liver taken ( $=Q_{O_2}$ , uncorr.). Figures in parentheses are maximum and minimum values.

effect is obtained even in the presence of 0.01 *M* malonate and has also been demonstrated in normal liver and kidney preparations. The optimum conditions for citrate oxidation have not yet been worked out but presumably isocitrate is the compound oxidized.

When isotonic saline homogenates are washed, the contents of the more fragile cells are washed away and the ability of the washed preparation to oxidize octanoate or citrate thus depends in part upon the percentage of cells which do not break during homogenization and washing. One way to test the extent of this loss is to measure the succinoxidase activity in the original homogenates with cytochrome present, and in the washed preparations with cytochrome omitted. In the latter instance, only the whole cells appear to oxidize succinate (12). The test is thus an indication of the fragility of the liver cells, although it is also a measure of the extent of cell disruption due to variations in the technique of the operator. In either case it provides objective data to aid in the interpretation of the data on the oxidation of octanoate and citrate. The succinoxidase data for the various groups of animals are reported in Table II. The average  $Q_{O_2}$  for the six normal rats (Group I) was 88.8. This is in excellent agreement with the value of 87.7 reported by Schneider and Potter (13) and the value of 87.5 reported by McShan *et al.* (14). It is seen that the loss due to homogenization and washing was about 50%, since the unfortified washed cells had an average  $Q_{O_2}$  of 42.1. Both the carbohydrate and the fat-fed rats showed sig-

nificant decreases in total succinoxidase activity and there was a suggestion that the liver cells from fat-fed animals were somewhat more fragile. On the other hand the livers from Group IV did not seem to differ significantly from Group I, with respect to succinoxidase activity.

TABLE II  
*Succinate Oxidation in Liver Preparations*

Group	No. of animals	Total Succinoxidase	Succinoxidase in washed cell,*
		$Q_{O_2}$	$Q_{O_2}$
I. Stock Diet	6	88.8 (65.2-115.6)	42.1 (14.2-63.5)
II. Carbohydrate	8	51.8 (45.4-63.6)	34.3 (17.6-53.3)
III. Fat	25	57.6 (29.2-92.0)	23.9 (5.1-46.0)
IV. Stock Diet, livers taken 20-30 minutes <i>post mortem</i>	2	71.6 (64.8-78.4)	44.2 (41.6-46.8)

\* Based on original homogenate with cytochrome added (12, 13).

\*\* Based on washed preparation with cytochrome omitted (12, 13), and referred to original quantity of liver.

Results reported as average  $Q_{O_2}$  (uncorr.) with maximum and minimum values in parentheses.

The data on citrate oxidation are in Table III. The differences between the various groups are slight and are of doubtful significance. They thus serve to emphasize the significance of the differences observed in the case of the other 2 substrates. The data are consistent in demonstrating the activating effect of potassium ions and in showing that lysed cells oxidize citrate more rapidly than whole cells in all groups.

TABLE III  
*Citrate Oxidation in Liver Preparations*

Group	No. of animals	Washed cells		Lysed cells	
		+Na <sup>+</sup>	+K <sup>+</sup>	+Na <sup>+</sup>	+K <sup>+</sup>
		$Q_{O_2}$	$Q_{O_2}$	$Q_{O_2}$	$Q_{O_2}$
I	6	5.9 (5.0-6.4)	6.3 (5.0-7.2)	8.7 (5.2-11.2)	10.5 (7.1-12.2)
II	5	3.6 (1.9-5.5)	4.5 (3.5-6.9)	7.1 (5.0-8.2)	9.2 (6.9-11.5)
III a	13	6.4 (5.0-7.7)			
b	12	5.2 (3.2-8.3)	6.0 (3.8-8.4)	7.3 (3.7-10.3)	8.4 (3.0-12.1)
IV	2	5.0 (5.0-5.0)	5.5 (5.2-5.8)	3.8 (2.4-5.2)	5.2 (3.4-7.0)

Each sample was studied with sodium ions only, or with sodium and potassium ions present as indicated (12). Data reported as average  $Q_{O_2}$  (uncorr.) with maximum and minimum values in parentheses.

The most interesting results were obtained using octanoate as the substrate (Table IV). When the capacity for octanoate oxidation was studied, using washed liver cells with either cation mixture, the data were roughly parallel to the rates on succinate. Lysis of the cells in the case of the *normal* rats (Group I) did not decrease the rate of octanoate

TABLE IV  
*Octanoate Oxidation in Liver Preparations*

Group	No. of animals	Washed		Lysed	
		+Na <sup>+</sup>	+K <sup>+</sup>	+Na <sup>+</sup>	+K <sup>+</sup>
		Q <sub>02</sub>	Q <sub>02</sub>	Q <sub>02</sub>	Q <sub>02</sub>
I	6	8.9 (5.5-12.5)	10.5 (6.9-12.6)	7.8 (2.1-10.8)	10.8 (0-14.7)
II	5	5.3 (2.8-6.9)	7.5 (6.6-8.1)	3.1 (2.3-4.0)	6.0 (3.3-7.6)
III a	13	5.6 (0-10.0)			
b	12	5.1 (0-8.9)	6.0 (0-9.7)	0.9 (0-3.8)	1.5 (0-6.4)
IV pm	2	7.4 (6.6-8.2)	9.6 (9.4-9.8)	1.3 (0-2.7)	1.7 (0.3-3.1)

Each sample was studied with sodium ions only, or with sodium and potassium ions present as indicated (12). Data reported as average Q<sub>02</sub> (uncorr.) with maximum and minimum values in parentheses.

oxidation when K<sup>+</sup> ions were present, in confirmation of the previous report (12). The livers from the rats fed carbohydrate (Group II) showed a slight loss of activity on lysis, and the potassium effect was more pronounced. However, in the case of the rats fed the high-fat diet (Group III) lysis of the cells caused a marked loss in activity, and K<sup>+</sup> ions were incapable of restoring the ability to oxidize octanoate. In many instances in this group the rate of oxygen uptake by the lysed preparations was zero, in marked contrast to the results from Groups I and II.

The results from Group IV (Table IV) represent an attempt to explain the data observed in Group III. Normal rats comparable to those in Group I were decapitated and the livers were allowed to remain anoxic *in situ* for various periods of time before they were removed, homogenized, washed and lysed in the usual manner. It was found that after a few minutes *post mortem*, the activity of the lysed preparations began to fall more rapidly than the activity of the whole cells, so that in specimens taken at 20-30 minutes *post mortem*, the data closely resembled that observed with animals from Group III.

These data are reported in Table IV. Longer periods of incubation resulted in loss of activity even in the whole cell preparations. The effect of anoxia is presumably to deplete the reservoirs of phosphate bond energy (15, 16) and to cause a progressive loss of phosphorylated coenzymes from the tissues, possibly first by a removal of phosphate groups and later by decomposition, and still later possibly the protein component may be lost. This process is probably initiated even during the normal manipulations prior to testing the activity. In the fatty acid oxidase system employed here, ATP was present in all cases. It would appear that at critical levels of phosphorylated cofactors, the added ATP is able to restore the system sufficiently so that activity

TABLE V

*Rate of Inactivation of Octanoate Oxidase System in Washed and in Lysed Liver Cells*

Expt. No.	Incubation time and temperatures prior to test	Oxygen uptake in test system	
		Washed cells	Lysed cells
		$\mu\text{l. O}_2$	$\mu\text{l. O}_2$
2-6	0 (Control)	59.2	55.7
	2.5 hours, 0°C.	54.0	42.8
	5.0 hours, 0°C.	54.0	22.9
2-14	0 (Controls)	70.6, 65.7	68.2, 68.0
	5 minutes at 38°C.	66.0, 61.6	36.5, 33.1
	5 minutes at 38°C., 1 mg. crude cozymase* in test system.	71.4	31.5
	5 minutes at 38°C., 3 mg. crude cozymase* in test system.	62.3	35.4

The results are expressed as the oxygen uptake per best consecutive 10 minutes (usually 2nd and 3rd 5-minute period) per 100 mg. of original fresh tissue.

\* The cozymase was kindly supplied by Merck and Co. Its activity was checked by simultaneous tests in the malic dehydrogenase system (10).

may be observed, but that further decomposition of essential components cannot be reversed by the addition of ATP. This explanation requires that the loss of essential components proceeds more rapidly in the lysed preparation than in the whole cell preparation. Data on this point are reported in Table V.

In carrying out the experiments reported in Table V, normal rats comparable to those in Group I were used. Washed liver cells from 10% isotonic saline homogenates were either suspended in saline or lysed in distilled water equivalent to the original

volume of homogenate and incubated at either 0° or 38°C. Aliquots of 1 ml. were taken at appropriate time intervals and tested for the activity of the octanoate system in the presence of all the known activators, including K<sup>+</sup> ions, and with equal amounts of NaCl in the final reaction mixtures. Two representative experiments are reported. The data show that in the lysed cells, marked losses in activity occur under conditions which cause essentially no loss in activity in washed cells from the same liver. It was also found that cozymase (Co I, DPN) was incapable of activating the system under these conditions, although activation by this coenzyme had been previously reported (11). The data suggest that the low activity in the lysed liver cells from anoxic or fat-fed rats might be due to a lower content of some cofactor. Another factor to consider is the extreme sensitivity of the system to calcium ions, which cause marked inhibition when added to the test system at final concentrations of 0.00025 M.

The experiments provide no evidence of an increase in the amount of the octanoate oxidase system in livers from animals fed a high fat diet. On the contrary, they suggest that these livers may contain decreased amounts of some cofactor which is necessary for octanoate oxidation. This finding is in line with the observations of Kaplan and Greenberg (17) who found that the ATP content of livers from fat-fed animals was greatly decreased in comparison with stock controls or carbohydrate-fed animals. However, our negative data could be due to the use of an inappropriate level of protein in the diet, to the improper length of time for keeping the animals on the diet, or to some other unknown factor. The data suggest that the octanoate oxidase system may require a cofactor other than ATP or DPN.<sup>6</sup>

### SUMMARY

1. The ability to oxidize succinate, citrate and octanoate was studied in livers from rats which were fed a high-fat diet for periods up to 21 days in comparison with livers from rats which received a high-carbohydrate diet, or a stock diet. In addition the enzyme tests were carried out on livers which had been anoxic *in situ* following decapitation.

2. Citrate was found to be oxidized by either whole or lysed liver cells in the presence of added adenosine triphosphate (ATP) but no significant dietary effects were noted.

<sup>6</sup> We have, on various occasions, attempted to assay liver tumors for this enzyme but have been unable to obtain evidence that the enzyme system is present, although it is possible that the tumor cells contain the enzyme but are deficient in a necessary cofactor. Recently Vestling *et al.* have reported that the livers of leukemic mice are unable to oxidize octanoate (18).



3. The succinoxidase activity was decreased in the high-fat and high-carbohydrate groups.

4. The octanoate oxidase activity was markedly decreased in lysed cell preparations of livers from the fat-fed group. Similar effects were noted in livers which had been anoxic 20–30 minutes. Preparations from the other groups showed little loss in octanoate oxidase activity when lysed.

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# The Purine Metabolism of Rat Liver and Kidney Slices *in vitro*

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## INTRODUCTION

The purine metabolism of mammalian tissue slices *in vitro* has been investigated by Reindel and Schuler (1). Their main conclusions are that hypoxanthine is not readily oxidized to uric acid by rat liver and kidney, and, moreover, that hypoxanthine reduces uric acid to xanthine under certain conditions. They also state that  $M/1,000$  cyanide inhibits the oxidation of xanthine to uric acid. No further work has been done on this subject, so it was of interest to repeat and extend these observations, especially in view of Quick's (2) results, which showed that the administration of pyruvic acid increased, and of lactic acid decreased, the excretion of uric acid in man. This suggests a possible interrelationship between purine and carbohydrate metabolism. A few experiments were also performed on the metabolism of certain methylxanthines in tissue slices.

## EXPERIMENTAL

Adult white rats were killed by a blow on the head, decapitated and bled. Liver and kidney slices were made in the usual way, and 300 mg. (wet weight) were suspended in 4.0 ml. Krebs' bicarbonate Ringer solution, equilibrated in 50 ml. Erlenmeyer flasks with 95%  $O_2$  and 5%  $CO_2$ , or with 95%  $N_2$  and 5%  $CO_2$ . At the end of the incubation period (usually 3 hours), 1.0 ml. of 20% trichloroacetic acid was added and the precipitated protein centrifuged down. The uric acid in a 1.0 ml. aliquot was estimated according to the method of Benedict and Franke (3) with the Folin molybdate-free reagent. After the addition of the reagent, the tubes were placed in a boiling water bath for exactly 3 minutes, cooled, diluted with 50 ml. of water and the color estimated in the Evelyn colorimeter with a 660 filter. The allantoin was estimated by the method of Young and Conway (4) on a 1.0 ml. aliquot which was neutralized before the reagents were added. A 490 filter was used. Estimations of uric acid and

of allantoin in liver and kidney slices at the beginning of the experiment showed that only traces were present. The values obtained at the end of the incubation period are, therefore, the result of the *in vitro* production of these substances.

When liver slices are incubated aerobically, with or without added purines, no uric acid is found at the end of the experiment, for the uricase present in the liver is able to oxidize all the uric acid formed to allantoin. After 3 hours incubation 0.20 mg. allantoin was formed in the control, and from 0.2 mg. each of hypoxanthine, xanthine and uric acid, 0.126, 0.163 and 0.182 mg. more allantoin was formed. Eighty to 90% of the allantoin added can be recovered at the end of the experiment. When kidney slices are incubated aerobically, uric acid accumulates and hypoxanthine and xanthine are quantitatively oxidized to uric acid. Added uric acid can be completely recovered. The formation of uric acid in the control and from the added purines can be inhibited 80% by *M*/250 quinimine, which has been shown to inhibit the xanthine oxidase (5), but not by cyanide.

When liver slices are incubated anaerobically, with or without cyanide, relatively large amounts of uric acid are produced, but only traces of allantoin are formed. It was first thought that the uric acid came from the splitting of some precursor such as the uric acid riboside described by Davis *et al.* (6). The addition of liver *kochsaff* or of yeast pentonucleotide increases the anaerobic uric acid and both these preparations might contain preformed uric acid in a bound form. However, the addition of xanthine increases the anaerobic production of uric acid. Either sufficient oxygen is present under our experimental conditions for the oxidation of xanthine to uric acid but insufficient for the oxidation of uric acid to allantoin, or xanthine may be oxidized by a hydrogen acceptor present in the liver. The addition of pyruvate was found to increase the amount of uric acid produced from xanthine anaerobically, and it can be assumed that the liver cells contain sufficient pyruvic acid to account for the oxidation of xanthine in the absence of added pyruvate. Pyruvic acid has no effect on the estimation of uric acid.

The pyruvic acid effect is much more marked in kidney slices. Anaerobically, only traces of uric acid are produced and the addition of xanthine causes a slight increase. Presumably the kidney cells contain little pyruvate, for upon its addition the uric acid produced by the control is greatly increased and xanthine is rapidly oxidized. In neither liver nor kidney can fumarate substitute for pyruvate. The

TABLE I

*The Effect of Various Factors on the Anaerobic Uric Acid Formation in 300 mg. (wet weight) of Rat Liver or Kidney Slices in 4.0 ml. Krebs' Bicarbonate Solution and 95% N<sub>2</sub> and 5% CO<sub>2</sub>. 3 Hours Incubation at 37°C.*

Tissue	Compounds added	Uric acid formed	Difference
		mg.	mg.
Liver	None	0.115	
	0.2 mg. Hypoxanthine	0.090	-0.025
	0.2 mg. Xanthine	0.165	0.050
	4.0 mg. Lactate	0.115	
	4.0 mg. Lactate+0.2 mg. Hypoxanthine	0.125	0.010
	4.0 mg. Lactate+0.2 mg. Xanthine	0.185	0.070
	4.0 mg. Pyruvate	0.148	
	4.0 mg. Pyruvate+0.2 mg. Hypoxanthine	0.311	0.163
	4.0 mg. Pyruvate+0.2 mg. Xanthine	0.366	0.218
	4.0 mg. Pyruvate+4.0 mg. Lactate	0.160	
	4.0 mg. Pyruvate+4.0 mg. Lactate+0.2 mg. Hypoxanthine	0.311	0.151
	4.0 mg. Pyruvate+4.0 mg. Lactate+0.2 mg. Xanthine	0.364	0.204
	0.1 mg. NaCN	0.110	
	0.1 mg. NaCN+0.2 mg. Hypoxanthine	0.070	-0.040
	0.1 mg. NaCN+0.2 mg. Xanthine	0.135	0.025
	0.1 mg. NaCN+4.0 mg. Pyruvate	0.162	
	0.1 mg. NaCN+4.0 mg. Pyruvate+0.2 mg. Hypoxanthine	0.322	0.160
	0.1 mg. NaCN+4.0 mg. Pyruvate+0.2 mg. Xanthine	0.385	0.223
	4.0 mg. Fumarate	0.112	
	4.0 mg. Fumarate+0.2 mg. Xanthine	0.163	0.051
Kidney	None	0.062	
	0.2 mg. Hypoxanthine	0.021	-0.041
	0.2 mg. Xanthine	0.072	0.010
	4.0 mg. Pyruvate	0.172	
	4.0 mg. Pyruvate+0.2 mg. Hypoxanthine	0.256	0.084
	4.0 mg. Pyruvate+0.2 mg. Xanthine	0.339	0.167
	4.0 mg. Pyruvate+0.2 mg. Xanthine+4.0 mg. Lactate	0.341	0.169
	4.0 mg. Fumarate	0.057	
	4.0 mg. Fumarate+0.2 mg. Xanthine	0.075	0.018

pyruvate effect may explain the results of Quick and it was, therefore, of interest to determine whether lactate has the opposite effect. Lactate, however, has no effect on uric acid production in liver or kidney, nor does it inhibit the action of pyruvate. There is, then, apparently no reversibility and no interaction of the lactic-pyruvic acid and xanthine-

TABLE II

*The Effect of Hypoxanthine on the Anaerobic Uric Acid Metabolism of 300 mg. (wet weight) of Rat Liver and Kidney Slices in 4.0 ml. Krebs' Bicarbonate Solution and 95% N<sub>2</sub> and 5% CO<sub>2</sub>. 3 Hours Incubation at 37°C.*

Tissue	Compound added	Uric acid formed	Difference
		mg.	mg.
Liver	None	0.117	
	0.2 mg. Hypoxanthine	0.126	0.009
	0.2 mg. Uric Acid	0.246	0.129
	0.2 mg. Hypoxanthine+0.2 mg. Uric Acid	0.260	0.143
	0.2 mg. Xanthine	0.185	0.068
	0.2 mg. Hypoxanthine+0.2 mg. Xanthine	0.130	0.013
Kidney	None	0.073	
	0.2 mg. Hypoxanthine	0.027	-0.046
	0.2 mg. Uric Acid	0.068	0.195
	0.2 mg. Hypoxanthine+0.2 mg. Uric Acid	0.217	0.144
	0.2 mg. Xanthine	0.093	0.020
	0.2 mg. Hypoxanthine+0.2 mg. Xanthine	0.078	0.005
	4.0 mg. Pyruvate	0.172	
	4.0 mg. Pyruvate+0.2 mg. Hypoxanthine	0.298	0.126
	4.0 mg. Pyruvate+0.2 mg. Uric Acid	0.382	0.210
	4.0 mg. Pyruvate+0.2 mg. Hypoxanthine+0.2 mg. Uric Acid	0.492	0.320
	4.0 mg. Pyruvate+0.2 mg. Xanthine	0.329	0.157
	4.0 mg. Pyruvate+0.2 mg. Hypoxanthine+0.2 mg. Xanthine	0.302	0.130

uric acid systems in a thermodynamic sense. Pyruvate, which acts equally well in the presence of cyanide, is therefore acting merely as a hydrogen acceptor. These results are shown in Table I.

Hypoxanthine behaves differently in some respects from xanthine. When it is added to liver or kidney slices anaerobically, with or without cyanide, it sometimes inhibits the uric acid formation in liver and

always does so in kidney. The variability of the effect in liver may depend on the amount of pyruvate present for, upon its addition, hypoxanthine is rapidly oxidized to uric acid. This also occurs in kidney. The inhibitory effect of hypoxanthine may be due to the fact that it reduces uric acid, as claimed by Reindel and Schuler, or that it interferes in some way with the oxidation of xanthine. When uric acid is incubated anaerobically with liver some of it disappears. As it does not form allantoin, it is presumably reduced to xanthine. The amount which disappears varies in different experiments from 10–50%. In no cases, however, have we observed that hypoxanthine increases the disappearance of added uric acid. On the other hand, hypoxanthine inhibits the production of uric acid from xanthine, and this occurs even in the presence of pyruvate. Thus, hypoxanthine interferes in some way with the production of uric acid but does not reduce pre-formed uric acid. It is possible that added uric acid may be reduced by substances other than hypoxanthine. Lactic acid is, however, without effect. In kidney, anaerobically, uric acid is not reduced with or without hypoxanthine but the production of uric acid from xanthine in the presence of pyruvate is inhibited by hypoxanthine. These results are shown in Table II.

When added to liver, adenine and guanine behave like hypoxanthine and xanthine, respectively, with the exception that adenine is slowly deaminated whereas guanine is rapidly converted to xanthine and produces uric acid or allantoin to the same extent as xanthine. *M*/3,000 sodium arsenite inhibits uric acid production 90% but *M*/360 theobromine is without effect.

#### *In Vitro Metabolism of Methyl Xanthines*

In connection with the purine metabolism of tissue slices, it was of interest to study the metabolism of the methyl xanthines. Myers and Hanzal (7) first showed that caffeine and theophyllin are oxidized and partially demethylated in the animal. It has been possible to show that this oxidation occurs with tissue slices. The livers, but not the kidneys, of the rat, guinea pig and rabbit oxidize caffeine and theophyllin. Because of the limitations of the method, it is impossible to decide whether caffeine is demethylated but it is probable that theophyllin is partially demethylated in position 3. The determination depends on the fact that 1,3-dimethyluric acid gives 72% of the color given by an

equimolar amount of uric acid, whereas 1-methyluric acid gives 85%. In about half of the experiments, demethylation occurred as well as oxidation; in the other half, the evidence for demethylation was equivocal. The system responsible for these reactions is very feeble. It requires 4-5 hours incubation with 300 mg. of tissue to oxidize 0.01 mg. of theophyllin. In this time, 0.02 mg. is only 70% oxidized, and 0.03 mg. 40%. Cyanide inhibits the oxidation completely and  $M/7,000$  theobromine inhibits 40%. The demethylation is not catalyzed by the sarcosine oxidase. Sarcosine does not inhibit the demethylation of theophyllin nor does the latter, in large concentrations, inhibit the sarcosine oxidase.

### DISCUSSION

Sixty mg. (dry weight) of rat liver slices in 95% oxygen produce approximately 0.05 mg. of allantoin/hour, and the same weight of kidney slices produces about the same amount of uric acid. Anaerobically, liver produces about half this amount as uric acid, but, when pyruvate is added, the uric acid production corresponds to the aerobic values for allantoin. Anaerobically, kidney produces very little uric acid, but the addition of pyruvate brings the value up to the aerobic figure. Since pyruvate apparently acts only as a hydrogen acceptor for hypoxanthine and xanthine, it is probable that the hydrolysis of nucleic acids proceeds at the same rate aerobically and anaerobically but that the final oxidation is regulated not only by the amount of oxygen available but also by the amount of pyruvate. That pyruvate can increase the uric acid excretion in man indicates that, *in vivo*, the oxygen tension may be a limiting factor in the production of uric acid under normal conditions, and that the amount of pyruvate available may be important in regulating the amount produced. Hypoxanthine, on the other hand, tends to inhibit uric acid production and acts in the opposite direction from pyruvate. These facts suggest that nucleic acids are hydrolyzed at a constant rate but that the oxidation of the final products to uric acid is regulated according to the needs of the cell.

### SUMMARY

1. Rat liver slices produce allantoin aerobically. The addition of hypoxanthine or xanthine increases this production.
2. Liver slices incubated without oxygen produce uric acid. Xanthine increases this production but hypoxanthine inhibits it. Under

anaerobic conditions pyruvate accelerates the oxidation of both hypoxanthine and xanthine to uric acid. Lactate and fumarate are without effect.

3. A variable amount of uric acid, added to liver slices anaerobically, disappears. Hypoxanthine has no effect on the rate of disappearance but markedly decreases the amount of uric acid formed from xanthine.

4. Kidney slices produce uric acid aerobically and this production is increased by hypoxanthine and xanthine.

5. Anaerobically, little uric acid is produced and hypoxanthine inhibits this. Pyruvate has the same effect as in liver.

6. Added uric acid can be recovered quantitatively from kidney slices. Hypoxanthine does not reduce added uric acid but decreases its formation from xanthine.

7. Theophyllin is oxidized and probably partially demethylated by liver but not by kidney slices. The properties of this reaction are briefly described.

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# Cobalt Metabolism Studies. III. Excretion and Tissue Distribution of Radioactive Cobalt Administered to Cattle <sup>1</sup>

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## INTRODUCTION

Cobalt metabolism investigations have become of interest due to widespread recognition of the economic importance of cobalt deficiency diseases in the live-stock industry, coupled with the fact that knowledge is scarce concerning the essentiality, behavior and rôle of cobalt in the animal. While there seems to be little question that cobalt is necessary in the ruminant diet, the situation in the case of non-ruminants is less clear. These considerations have led to emphasis on experimentation with cattle, although similar data for rats, rabbits, and swine have been obtained and will be reported elsewhere.

The literature on cobalt in relation to animal health has been comprehensively reviewed to 1944 by Russell (1). In 1941 Copp and Greenberg (2) demonstrated the use of radioactive cobalt with rats, and in 1943 Greenberg, Copp and Cuthbertson (3) extended this work to show the appearance of labeled cobalt in the bile, urine, feces and liver of bile-fistula rats. Sheline, Chaikoff and Montgomery (4) followed intravenously injected radioactive cobalt in the plasma, bile and pancreatic juice of dogs provided with bile and pancreatic fistulas. Houk, Thomas and Sherman (5) have recently published on the interrelationships of dietary iron, copper and cobalt in the metabolism of rats, as indicated by chemical analysis of the feeds and the carcasses. In 1946 Comar, Davis and Taylor (6) reported on radioactive cobalt proce-

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dures with rats and cattle, and presented data on the excretion of cobalt by rats and preliminary findings on the tissue distribution of cobalt administered to cattle. Comar, Davis, Taylor, Huffman and Ely (7) have recently shown the partition of radioactive cobalt administered to a rumen fistula cow.

This paper describes an improved technique for the measurement of radioactive cobalt and presents data on the excretion and tissue distribution of labeled cobalt injected and fed to cattle. The transmission of cobalt from dam to fetus is demonstrated.

### EXPERIMENTAL

*Radioactive Material.* The radioactive cobalt was supplied by the Massachusetts Institute of Technology Radioactivity Center and was prepared by bombardment of iron with deuterons. It was obtained as a purified solution of cobalt chloride and consisted of a mixture of three cobalt isotopes ranging from 65 to 270 days half life with the average falling near 80 days. Three preparations have been received to date for use in this work. *Sample 1* contained 9 mg. cobalt with an initial specific activity of 267  $\gamma$  radium gamma-ray equivalent/mg., *Sample 2* contained 5.45 mg. cobalt with an initial specific activity of 3303  $\gamma$  radium gamma-ray equivalent/mg. and *Sample 3* contained 3 mg. cobalt with an initial specific activity of 10,680  $\gamma$  radium gamma-ray equivalent/mg. In the case of *Sample 1* the measurements were sensitive to 0.004  $\gamma$  cobalt 45 days after its preparation; with *Sample 2* the sensitivity was 0.0006  $\gamma$  after 20 days; and with *Sample 3* the sensitivity was 0.0001  $\gamma$  after 76 days ( $\gamma$  represents micrograms).

*Measurement of Radioactivity.* Some of the data presented here were obtained by the method reported in detail earlier (6). This procedure consisted essentially of ashing the sample, bringing the cobalt into solution and quantitatively electroplating the metal onto a copper disk which was used for the actual measurement with Geiger-Mueller Counter Apparatus. However, it was found advantageous to make the measurements in solution, employing a dipping type counter tube (purchased from Distillation Products, Inc., Rochester, New York). This tube is constructed similarly to that described by Bale, Haven and Lefevre (8) and is operated at about 1200 volts. The background, due primarily to cosmic radiation, averaged 12 counts/minute. Each solution was measured for 10 minutes, or longer, depending on the activity contained, and net counts of 4/minute or less were considered as not significant. This tube was used for cobalt in the same manner as recently described by Comar and Neller (9) for the measurement of radioactive phosphorus in solution, except that the cells were supported in a plastic holder instead of wax. Cells containing as little as 2 ml. of the unknown solution can be used to give maximum sensitivity. However, for the work reported here, 15 ml. cells were used. The sensitivity was decreased about three-fold by using the 15 ml. instead of the 2 ml. cells. The sensitivity with the 15 ml. cells was about the same as that obtained when the electroplating procedure was employed. For measurement in solution, the samples were ashed and the ash taken up in 2 *N* hydrochloric acid as earlier described. The acid solution was

then made to volume with distilled water and the measurements made directly on this solution. The calibration curve was obtained by adding known amounts of the radioactive cobalt preparation to inert samples and carrying them through the same procedure.

The use of the dipping type counter tube has greatly facilitated this measurement since many of the time-consuming steps necessary in the electroplating procedure are eliminated.

### EXPERIMENTAL RESULTS AND DISCUSSION

*Excretion of Cobalt by Cattle.* The excretion data in Table I were obtained with purebred Jersey steers, about 3 years old and weighing 600-700 pounds. They were normal animals in good condition, and were on an adequate diet consisting of corn, ammoniated citrus pulp and cottonseed meal, supplemented with bone meal and shark liver oil; a copper, cobalt and iron drench was given periodically.

A simple digestion stall (plans obtained from Dr. J. K. Loosli) was used for the quantitative collections of feces and urine, which were made daily. The steers were placed in the stalls at least 10 days before the start of the experiment, and the cobalt was not administered until the animals were consuming normal amounts of feed. The cobalt solution was fed by capsule or injected into the jugular vein.

TABLE I

*Excretion of Labeled Cobalt by Cattle, in Percent of Administered Dose  
(218  $\gamma$  Cobalt)*

Administration	No. of animals	Feces	Urine
Oral	3	78.3 $\pm$ 3.2*	0.62 $\pm$ 0.08
Injection	2	32.5 $\pm$ 0.8	61.6 $\pm$ 4.8

\* The mean value  $\pm$  the mean deviation.

In a previous paper (7) values for the excretion of cobalt by a rumen fistula cow were given which were based on the concentration in the samples taken and an estimate of the daily excretion. The data in Table I are, therefore, considered the more reliable although the agreement is fair in most respects.

Very little of the orally administered cobalt was available for distribution throughout the tissues as indicated by the small amount appearing in the urine; this is supported by data presented later, on the actual tissue concentrations obtained. The cobalt first appeared in

the feces 15-24 hours after ingestion and most of the feces elimination occurred between 24 and 72 hours; very little was excreted after 7 to 9 days. The rate of passage of cobalt through the digestive tract was similar to that reported for inert materials by Moore and Winter (10).

When the cobalt was injected, about 62% appeared in the urine and 33% in the feces. Significant amounts of cobalt were found in the urine 40 minutes after injection with most of the urinary excretion occurring within the first 36 hours. The rate of appearance of injected cobalt in the feces was about the same as in the case of oral administration. Using the values reported later for the amounts of injected cobalt found in the bile, together with an estimate of the daily biliary secretion, it was calculated that 5-15% of the injected cobalt was probably eliminated via the bile. Sheline, Chaikoff and Montgomery (4), working with dogs, found that 5% of the intravenously injected cobalt was contained in the bile collected during approximately 72 hours.

*Tissue Distribution in Cattle.* In this study it was considered desirable to use animals reared on a cobalt-deficient diet in addition to those maintained on an adequate ration. Two steers, from the group described above as employed in the excretion experiments, were used to represent the normal animal.

Steer No. 8, about 3 years old, weight 600 lbs.

Steer No. 9, about 3 years old, weight 585 lbs.

The other animals listed below, were range cattle, brought to Gainesville from an area in Florida considered deficient in some of the essential minerals. They were then fed on a ration which contained less than 0.01 p.p.m. cobalt by analysis, and which consisted of redtop hay harvested from a low cobalt area, corn grown on a low cobalt area, a protein supplement of dried skim milk, and a phosphorus supplement of tricalcium phosphate. For about 6 months, under these conditions, most of the animals remained in poor condition and gave an appearance of emaciation, rough hair coat and extreme weakness.

Steer No. 1, about 2 years old, weight 250 lbs.

Steer No. 2, about 2 years old, weight 230 lbs.

Heifer No. 3, about 1½ years old, weight 195 lbs.

Cow No. 4, about 10 years old, weight 400 lbs.

Cow No. 6, about 5 years old, weight 300 lbs.

Cow No. 7, about 8 years old, weight 525 lbs.

The procedure for the slaughter of the animals and the handling of the tissues has been described earlier (6). As a rule 50-100 g. samples were used, although when little activity was expected samples up to 500 g. were taken.

Table II presents data on the distribution of radioactive cobalt orally administered and injected into cattle which were then sacrificed after varying time intervals. The actual dosage employed was determined by the activity of the preparation at the time of administration. To make the values comparable they have been calculated on the basis of a 100  $\gamma$  cobalt dosage; this should not cause distortion of the data since the amounts of cobalt used were all relatively small. The sensitivity varied with the radioactive preparation employed and its age, and this value is, therefore, given for each set of measurements. All of the animals, with the exception of Cow No. 4, were given a single dose and sacrificed after the stated time; Cow No. 4 received 17.4  $\gamma$  cobalt twice a week for about a month, and was sacrificed 6 days after the last dose.

Consider the values for Cow No. 7, Steer No. 9 and Steer No. 1: Very little of the cobalt was available for general distribution throughout the tissues, which is in accord with the small urinary excretion of ingested cobalt as discussed above. The activity in the intestinal lymph glands indicated the probable route of absorption and was not surprising in view of the corresponding high values found in the injection studies. It must be remembered that, with some organs, negative results will be due in part to the small sample available; this is particularly the case with the pituitary, thyroid and adrenals.

The values for the gastro-intestinal tract and contents represent the cobalt on its way to excretion and the uptake by those tissues in contact with it. The disappearance of cobalt from the rumen contents as demonstrated with a rumen fistula cow has been reported elsewhere (7), however, the present data yield information on the concentrations in the other stomach compartments. With Cow No. 7, sacrificed after 16 hours, the percentages of the dose found in the contents of the abomasum, reticulum, omasum and rumen were 0.85, 1.6, 10.1 and 63.8, respectively. The corresponding values for Steer No. 9, sacrificed after 5 days, were 0.07, 0.09, 1.0 and 0.6. In the case of Cow No. 7, the tissue concentrations in the abomasum, reticulum, omasum, rumen, small intestine and large intestine were about the same with the small intestine showing the highest and the omasum showing the lowest

TABLE II

*Distribution of Labeled Cobalt in Cattle in  $\gamma/100$  g. Fresh Weight  
(based on 100  $\gamma$  dosage)*

	Oral administration				Jugular injection			
	Cow No. 7	Steer No. 9	Steer No. 1	Cow No. 4	Cow No. 6	Steer No. 8	Steer No. 2	Heifer No. 3
Actual dosage ( $\gamma$ Co)	218	60	1330	157	260	300	1330	2400
Sensitivity ( $\gamma$ Co)	0.0015	0.0001	0.01	0.001	0.001	0.003	0.01	0.01
Sacrificed after	16 hours	5 days	10 days	1 month <sup>1</sup>	2 hours	16 hours	24 hours	10 days
Tissue								
Pituitary	*	*	*	*	0.015	0.013	*	*
Thyroid	*	*	*	*	0.23	0.083	0.50	0.027
Thymus	*	*	*	0.0015	0.52	0.047	0.083	0.0036
Adrenals	*	*	*	0.0083	0.88	0.19	0.65	0.13
Reproductive organs	*	*	*	*	0.026	0.035	0.045	0.016
Brain	*	*	*	*	0.0065	0.0050	0.0062	0.0083
Eye	*	*	*	*	0.021	0.010	0.015	0.0046
Intestinal lymph glands	*	0.0035	0.0025	*	1.0	0.073	0.12	0.032
Salivary glands	—	*	—	—	0.17	—	—	0.015
Heart	*	*	*	*	0.10	0.041	0.073	0.014
Blood	*	*	*	*	0.12	0.066	0.037	0.013
Aorta	*	*	*	*	0.073	0.033	—	0.018
Lung	*	*	*	*	0.13	0.13	0.11	0.039
Trachea	*	*	*	*	0.086	0.028	0.14	0.026
Kidney	*	0.0056	*	*	0.29	0.22	0.26	0.059
Kidney fat	*	*	0.0038	*	—	0.080	—	—
Bladder	*	*	*	0.0028	0.081	0.033	0.25	0.015
Urine (not excreted)	0.0025	*	—	*	0.86	0.087	0.40	0.050
Tongue	*	*	*	*	0.054	0.027	0.037	0.0046
Esophagus	*	*	*	*	0.053	—	0.098	0.027
Abomasum	0.0055	0.0017	0.00098	*	0.12	0.087	0.098	0.0096
Abomasum contents	0.058	0.0080	*	*	0.13	0.023	0.0029	0.0054
Reticulum	0.0058	0.0016	—	*	0.073	0.033	0.045	0.010
Reticulum contents	0.20	0.0085	—	*	0.00035	0.0010	*	*
Omasum	0.0012	*	—	*	0.046	0.046	0.045	0.0083
Omasum contents	0.18	0.032	—	*	*	*	*	*
Rumen	0.0034	0.0053	*	*	0.062	0.035	0.053	0.0032
Rumen contents	0.21	0.0042	*	*	0.0015	0.0010	*	*
Small intestine	0.0064	0.018	0.0022	*	0.18	0.063	0.098	0.020
Small intestine contents	0.13	0.010	0.0035	*	0.32	0.046	0.038	0.0075
Large intestine	0.0029	0.002	*	*	0.096	0.043	0.11	0.0087
Large intestine contents	0.41	0.044	*	*	0.0025	0.15	0.03	0.0050
Pancreas	0.013	0.0027	—	—	0.59	0.22	0.47	*
Spleen	*	0.0027	0.0083	*	0.092	0.069	0.045	0.022
Liver	0.0012	0.013	0.020	0.0039	1.95	1.80	0.59	0.092
Gall bladder	*	*	*	0.0023	0.38	—	0.51	0.024
Bile	*	*	*	*	0.64	0.080	0.21	0.00088
Tenderloin muscle	*	*	*	*	0.049	0.0077	0.045	0.0015
Gastrocnemius muscle	*	*	*	*	0.031	0.0080	*	0.0041
Ligament (nuchal)	*	*	*	*	0.051	0.017	0.033	0.016
Cartilage (costal)	*	*	*	*	0.050	0.021	0.045	0.010
Bone (femur)	*	*	*	*	*	*	0.0033	0.0037
Red bone marrow (rib)	*	*	*	*	0.069	0.003	0.035	0.032
Wht. bone marrow (long bones)	*	*	*	*	0.0098	0.006	0.035	0.0087
Teeth	*	*	*	*	*	0.012	0.039	0.0021

<sup>1</sup> Cow No. 4 received 17.4  $\gamma$  twice weekly for 1 month; sacrificed 6 days after last dose.

\* Radioactivity measurements made but amount in sample less than value given for sensitivity.

values. With Steer No. 9, the differences were somewhat accentuated: the abomasum, reticulum and large intestine had about the same concentration, the rumen was slightly higher, while the small intestine had 5-10 times this activity, and the omasum showed no activity at all.

In the animal sacrificed 16 hours after cobalt ingestion, the pancreas had the highest concentration of all the organs, while, after 5 days, the pancreas concentration was about the same as that for the stomach tissues and was lower than that of the liver; after 10 days no activity was detected in the pancreas. On the other hand, the spleen showed no activity after 16 hours, but did have increasing values after 5 and 10 days, respectively, although the concentration was in each case lower than that of the liver. The percentages of the dose found in the livers of Cow No. 7, Steer No. 9 and Steer No. 1 were 0.024, 0.42 and 0.25, respectively.

In the case of Cow No. 4, which received small doses over a period of a month, it is interesting to note that there was some accumulation in the thymus and adrenals and that the liver retained about 0.1% of the total amount of cobalt administered. No activity was detected in the gastro-intestinal tract and contents. The significance of the activity found in the bladder and gall bladder is obscure.

Consider now the values given for the animals receiving cobalt by injection. It is apparent that the cobalt reached practically every tissue and organ. The rapid disappearance of injected cobalt from the blood has been discussed in a previous publication (7). The cobalt remaining in the blood was found almost exclusively in the plasma. As indicated by the excretion data, removal from the blood for urinary elimination was rapid, and this is confirmed by the relatively high concentrations found in the kidney and in the retained urine at the time of autopsy. Considerable and rapid deposition occurred in the liver as shown by the percentages of the dose found in the livers which were 30, 46, 7 and 1 after 2 hours, 16 hours, 24 hours and 10 days respectively. As discussed earlier, the high concentrations found in the bile indicate that this is a significant route of excretion.

The higher concentrations were consistently found in the glandular organs, particularly the adrenals, thyroid, liver, thymus, intestinal lymph glands and pancreas.

The fact that significant amounts of injected cobalt reached the abomasum contents, while very little was found in the rumen, omasum and reticulum contents, has been pointed out previously (6). The



concentration in the abomasum itself was higher than in the other stomach tissues, except with Heifer No. 3, in which case the abomasum and the reticulum had about the same value. With the exception of Steer No. 2, the concentration in the small intestine was higher than that in the large intestine. The cobalt accumulation in the muscle tissue was small and, with the exception of Cow No. 6, was less than that in the ligament and cartilage. There seemed to be no particular accumulation in the spleen and red bone marrow as might be expected were the cobalt concerned in hemoglobin formation. Small amounts were found in the teeth of the animals sacrificed after 16 hours, and in the bones of those sacrificed after 24 hours; the values indicated that, although accumulation was slow, the cobalt that did reach the teeth and bones was retained to a greater degree than in the case of most of the other tissues.

The rate of disappearance of cobalt from the various tissues may be compared by consideration of the values for Cow No. 6, sacrificed after 2 hours, and Heifer No. 3, sacrificed after 10 days. The following tissues showed about a 100-fold decrease in concentration: pituitary, thymus and pancreas. The following showed a 10- to 30-fold decrease: thyroid, intestinal lymph glands, salivary glands, heart, blood, tongue, abomasum, rumen, small intestine, large intestine, liver, gall bladder and muscle. The following decreased only 2- to 7-fold: adrenals, reproductive organs, eye, aorta, lung, trachea, kidney, bladder, esophagus, reticulum, omasum, spleen, ligament, cartilage and red bone marrow. The following remained about the same or showed a slight increase: brain, bone, white bone marrow and teeth.

It may be noted that the values for Steer No. 8, on an adequate diet, were in many cases lower than those for Cow No. 6 and Steer No. 2. This suggests that perhaps the uptake by Steer No. 8 was decreased due to the presence of a normal cobalt level in the body.

*Transmission of Cobalt from Dam to Fetus.* It was of interest to determine the extent to which cobalt fed to the pregnant cow could be transmitted to offspring and thereby constitute a reserve to be drawn upon after birth. Askew and Dixon (11) reported indications that cobalt was transmitted through the ewe to the offspring, but their data were admittedly inconclusive.

Two dairy animals were used for the oral administration part of this study. These cows were normal, in good condition and were on the regular dairy herd ration of pasture, corn silage, a limited amount of

alfalfa hay and mixed concentrates, with access to iron, copper, cobalt, salt and bone meal. One of them was a registered Jersey and was bred December 29, 1944; this animal was fed a total of 171  $\gamma$  radioactive cobalt in small doses given twice weekly from July 17 to September 27, 1945. The calf, weighing 61½ pounds, was dropped October 2 and was taken immediately for autopsy. The other cow was a grade Holstein bred February 21, 1945, to a Jersey bull; this animal was fed a total of 217  $\gamma$  radioactive cobalt in small doses given twice weekly from July 17 to November 21, 1945. The calf, weighing 59½ pounds, was dropped during the night of November 29 and was taken for autopsy early the next morning.

Tissue analyses, similar to those reported in Table II, were made on the calves. However, no activity was found except in the livers. The liver of the Jersey calf contained 0.016% of the total cobalt dose, while the liver of the grade Holstein calf contained 0.0067% of the dose.

For the injection study one of the range cows, on the ration described earlier, was used. This animal received a total of 218  $\gamma$  radioactive cobalt, injected in small doses into the jugular vein once a week from August 22 to October 24, 1945. Stillborn twins, weighing about 8½ pounds each and estimated to be about 6 months along, were dropped on October 25. Here, again, no activity in the tissues was found except as follows: In fetus No. 1 the kidneys contained 0.0018% of the total dose, the small intestine and contents contained 0.0087% and the liver contained 0.0096%. In fetus No. 2 the kidneys had 0.0020%, the small intestine and contents had 0.0055%, the large intestine and contents contained 0.0073%, and the liver had 0.014% of the total dose.

It is apparent that cobalt administered to the pregnant cow is transmitted across the placenta, primarily for storage in the liver of the fetus. The amount which reaches the fetus, however, is so small that its significance as a reserve is open to question.

#### ACKNOWLEDGMENT

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## SUMMARY

1. A procedure for the measurement of radioactive cobalt in solution is described. With the best preparation available so far the initial measurements were sensitive to 0.0001  $\gamma$  cobalt.

2. When labeled cobalt was administered orally to cattle about 80% of the dosage was eliminated in the feces and about 0.5% in the urine; very little was absorbed and available for general distribution throughout the tissues. Relatively high concentrations in the small intestine and intestinal lymph glands indicated the probable route of absorption. The liver was the chief storage organ; the liver of an animal sacrificed 5 days after cobalt ingestion contained 0.4% of the dose.

3. When labeled cobalt was injected into the jugular vein of cattle, the disappearance from the blood was rapid, and about 65% of the dose was eliminated in the urine and about 30% in the feces. There was general distribution throughout the tissues; considerable and rapid deposition of cobalt, up to 46% of the dose, occurred in the liver with subsequent excretion via the bile. The higher concentrations were found in the glandular organs, particularly the adrenals, thyroid, liver, thymus, intestinal lymph glands and pancreas. Small amounts of injected cobalt were found in the abomasum contents and practically none in the contents of the other stomach compartments.

4. Extremely small but definite amounts of radioactive cobalt injected or fed to pregnant cows were transmitted across the placenta for storage in the liver of the fetus.

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# The Germicidal Action of Bromine \*

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## INTRODUCTION

Simple controlled systems, which permit the separation of the several environmental factors affecting the germicidal action of chlorine, have been used effectively by Weber and Levine (1) and Butterfield *et al.* (2) for clarifying the complex reactions involved in killing bacteria with chlorine. Wyss *et al.* (3) have used similar techniques to show wherein the behavior of iodine differs from that of chlorine. The halogen bromine has not been thoroughly investigated by such techniques although McCarthy (4) and others (5, 6) have presented a study of bromine action in water and sewage. From their work it is evident that the effectiveness of bromine as a germicidal agent is influenced by environmental conditions in a manner no less complex than exists with the other halogens. Our experiments are designed to clarify the effect of a variety of conditions on germicidal activity and to compare this behavior with that of other halogens.

## METHODS

The bacteriological methods have been previously described (7). Essentially they involve the determination of the time required for killing 99% of the bacterial spores or vegetative cells in buffered solution which has been treated to render it completely free from oxidizing or reducing substances. Bromine concentrations were determined by iodometric titration in acid solution. Washed spores from an actively proteolytic strain of *Bacillus subtilis* were used in the sporicidal experiments although a few parallel experiments were carried out with *Bacillus metiens*. Vegetative cells of the

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spore-former were cultured on nutrient agar containing 0.1% glucose and harvested after incubating 24 hours at 37°C. The *Pseudomonas fluorescens* culture was isolated from spoiled meat; the cell suspensions were prepared daily from cells washed from agar slants. A concentrated bromine stock solution was prepared and added to the buffered test solutions and allowed to equilibrate before the addition of the bacterial spores or cells. Initial and final titrations were made in all experiments and only those data are reported where no great loss of titratable halogen occurred during the time required for the bactericidal action.

## RESULTS

The effect of changes in concentration on the killing action of bromine on *B. subtilis* spores was determined at 25°C. and at pH 7.0. The results of a series of experiments summarized in Fig. 1 show that

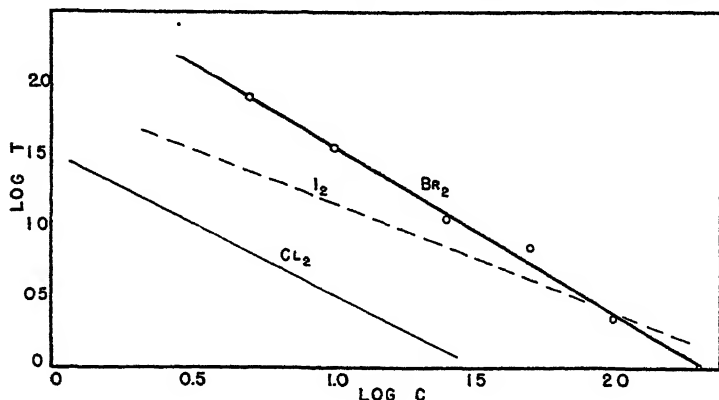


Fig. 1. Effect of Concentration on Killing Time by Bromine. Graphs representing chlorine and iodine activity under similar conditions are included for comparison (3, 7). Data were collected at 25°C. and pH 7.0.

with between 5 and 100 p.p.m. bromine a straight line is obtained when the log of the concentration (in p.p.m.) was plotted against the log of the time (in minutes) required for 99% killing. Results for concentrations of bromine less than 5 p.p.m. are not included since such experiments were complicated by high percentage decomposition of the bromine in the comparatively long time intervals required. A few experiments with *B. metiens* spores showed the resistance to be, within experimental error, equal to that of the *B. subtilis* spores. Fig. 1 includes for comparison the plot of iodine and chlorine concentration curves of *B.*

*metiens* spores obtained from the literature (3, 7). The best straight line for the bromine data is described by the formula

$$\log t = -1.23 \log c + 2.75.$$

The slope of the curve is greater than that observed either with chlorine or iodine indicating a greater change in activity with a given change in concentration.

The effect of temperature on the activity of bromine is shown in Table I. The activity increases slightly more than two-fold with each

TABLE I  
*Effect of Temperature on the Killing of B. subtilis Spores  
at pH 7.0 by 25 p.p.m. Bromine*

Temperature °C.	99% Killing time minutes
35	4
25	10
20	16
15	21
10	37
5	54

10°C. increment in temperature; thus the temperature characteristic is lower than that reported for iodine but higher than that reported for chlorine.

Within the pH range of biological experiments two equilibria must be considered, the hydrolysis equilibrium,



and the ionization of hypobromous acid,



The amounts of each bromine-containing species existing at pH values ranging from 3.0 to 10.0 are plotted in Fig. 2. The times required for 99% killing by 25 p.p.m. bromine at 25°C. at the indicated pH values are described by a double sigmoid curve.  $\text{Br}_2$  is the most active component, being about 10 times as active as HOBr. Above pH 3 the fraction present as  $\text{Br}_2$  decreases and HOBr is formed, which results in decreased activity as reflected by the increased killing time. Between pH values of 6 and 8, where most of the bromine is present as HOBr, there is little change in activity. Above pH 8, HOBr dissociates into

$OBr^-$  and activity drops sharply; therefore, like hypochlorite and hypiodite ions, hypobromite ion is essentially inactive.

At pH 9 the activity of bromine is sufficiently low that it is possible to measure with reasonable accuracy the killing of vegetative cells under the same experimental conditions employed with the spores. Washed vegetative cells of *B. subtilis* and washed cells of *P. fluorescens* gave concentration curves similar to those obtained with spores but were about 500 times less resistant.

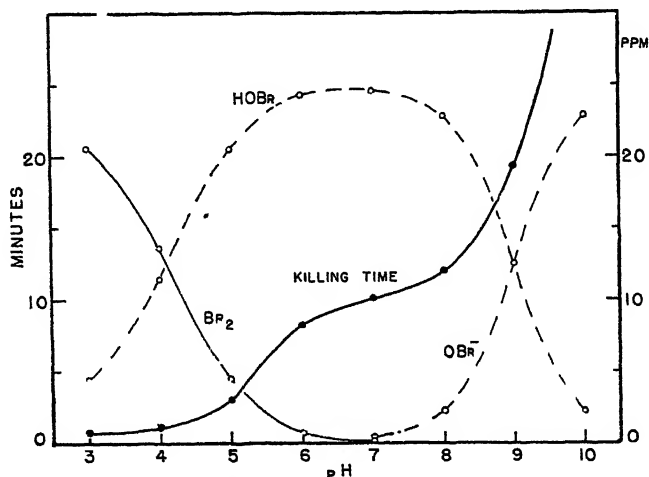


FIG. 2. Effect of pH on Killing Time and Distribution of Br. Experiments were conducted at 25°C. with 25 p.p.m. added  $Br_2$ . All concentrations are expressed in terms of  $Br_2$ .

Since the presence of ammonia is a major factor in determining the effectiveness of chlorine, its influence on the germicidal action of bromine was also investigated. Low concentrations of ammonia seriously impair the stability of bromine and consequently make it difficult to determine the germicidal action. At higher concentrations of ammonia (25 times as much ammonia as bromine) stable bromamines are formed which have germicidal activity equal to that of bromine in the absence of ammonia (Table II). Thus, while iodine is unaffected by  $NH_3-N$  and chloramines are much less active than free chlorine (3, 7), bromamines are stable only in the presence of excess ammonia and, under such conditions, they appear to be as active as free bromine.

TABLE II

*Effect of  $\text{NH}_3\text{-N}$  on the Killing of *B. subtilis* Spores  
at pH 7.0 by 20 p.p.m. Bromine*

$\text{NH}_3\text{-N}$ p.p.m.	10 Minute bromine residual p.p.m.	99% Killing time minutes
0	20	14
1	16	19
2	2	>100
3	2	>100
10	8	85
30	9	70
100	14	18
500	19	14
1000	19	14

## SUMMARY

The bactericidal action of bromine above pH 6 is due primarily to HOBr, and at lower values the more active  $\text{Br}_2$  is responsible for most of the activity. Bromine concentration curves for killing are not greatly different from those encountered with the other halogens. The effect of temperature is greater than that reported for chlorine but less than that for iodine. Vegetative cells are about 500 times more sensitive than the spores under the experimental conditions employed. In the presence of large excess of ammonia-nitrogen the killing action of bromine is not impaired.

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# The Theory of Isotope Dilution

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Isotope tracer technique is based on the use of compounds which have been enriched with respect to a given isotope ( $H^2$ ,  $C^{13}$ ,  $N^{15}$ , etc.). In many biochemical applications, the enriched compound, containing stably-bound isotope, is employed as a substrate for the metabolic processes of isolated tissues or intact organisms. The tracer isotope may then be found in greater than normal abundance in other compounds eventually isolated. From this type of information, inferences may be drawn regarding the metabolic pathways which relate the substrate with various intermediate and final products.

A second type of experiment is one in which isotopically normal substrate is already present in (or is produced by) the system to which enriched ("isotopic") compound is added. If uniform mixing occurs and if equilibrium is attained, the "isotopic" compound will undergo a predictable dilution with respect to the tracer isotope.

In the following, we shall be concerned with experiments of the second kind. This method has been developed as a useful analytical tool by Rittenberg and others, who employed it for the quantitative determination of amino acids in protein hydrolyzates and of fatty acids in animal fat preparations (1, 2, 3, 4).

## I. THE EQUATIONS OF ISOTOPE DILUTION FOR ELEMENTS

We begin by considering a simple case, viz., the result of mixing two batches of an element, each containing two isotopes in different proportions.

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The notation is as follows:

	Batch 1	Batch 2
Atom-% of isotope $A$	$A_1$	$A_2$
Atom-% of isotope $B$	$B_1$	$B_2$
Number of $A$ atoms	$N_{a1}$	$N_{a2}$
Number of $B$ atoms	$N_{b1}$	$N_{b2}$
Total number of atoms	$N_1$	$N_2$
Total mass (in g.)	$Y_1$	$Y_2$

Assuming  $A$  to be the tracer isotope, we shall determine the atom-% of  $A$  in the final mixture.

The following relations hold among the quantities defined above:

$$A_1 = 100N_{a1}/N_1; \quad B_1 = 100N_{b1}/N_1, \quad (1)$$

$$A_2 = 100N_{a2}/N_2; \quad B_2 = 100N_{b2}/N_2, \quad (2)$$

$$N_{a1}m_a/L + N_{b1}m_b/L = Y_1, \quad (3)$$

$$N_{a2}m_a/L + N_{b2}m_b/L = Y_2, \quad (4)$$

where

$m_a$  = atomic weight of isotope  $A$ ,

$m_b$  = atomic weight of isotope  $B$ ,

$L$  = Avogadro's number,

$$A_1 + B_1 = A_2 + B_2 = 100, \quad (5)$$

$$N_{a1} + N_{b1} = N_1; \quad N_{a2} + N_{b2} = N_2. \quad (6)$$

From equations (1)-(4), we obtain

$$N_{a1} = A_1 Y_1 L / R_1, \quad (7)$$

$$N_{a2} = A_2 Y_2 L / R_2, \quad (8)$$

$$N_{b1} = B_1 Y_1 L / R_1, \quad (9)$$

$$N_{b2} = B_2 Y_2 L / R_2, \quad (10)$$

where

$$R_1 = A_1 m_a + B_1 m_b, \quad (11)$$

$$R_2 = A_2 m_a + B_2 m_b. \quad (12)$$

By definition, the atom-% of isotope  $A$  in the final mixture is given by:

$$A_f = 100(N_{a1} + N_{a2}) / (N_1 + N_2). \quad (13)$$

Substituting from equations (7)-(10), and making use of the relations (5) and (6), we obtain:

$$A_f = (A_1 Y_1 R_2 + A_2 Y_2 R_1) / (Y_1 R_2 + Y_2 R_1). \quad (14)$$

A quantity frequently used in place of atom-% is atom-% excess, *i.e.*, atom-% excess above the normal abundance.

Letting  $A_0$  be the normal abundance of isotope  $A$  expressed in atom-%, and denoting the atom-% excess by the letter  $C$  with appropriate subscripts:

$$C_1 = A_1 - A_0, \quad (15)$$

$$C_2 = A_2 - A_0, \quad (16)$$

$$C_f = A_f - A_0. \quad (17)$$

Introducing equations (15)–(17) into (14) we get:

$$C_f = (C_1 Y_1 R_2 + C_2 Y_2 R_1) / (Y_1 R_2 + Y_2 R_1). \quad (18)$$

A particularly simple case is one in which  $m_a$  and  $m_b$  can be considered approximately equal. Then equations (14) and (18) reduce to:

$$A_f = (A_1 Y_1 + A_2 Y_2) / (Y_1 + Y_2), \quad (19)$$

$$C_f = (C_1 Y_1 + C_2 Y_2) / (Y_1 + Y_2). \quad (20)$$

The discrepancies which occur using isotopes with nearly equal masses are illustrated in Table I which lists the solutions to both equations (14 and 19) for 3 arbitrary sets of conditions

TABLE I  
*Comparison of Exact and Approximate Equations for  
Isotopes of Nearly Equal Mass*

Conditions	Equation (14) (rigorous)	$A_f$	Equation (19) (simplified)
1. $Y_1 = 10, A_1 = 20$ $Y_2 = 5, A_2 = 30$	23.359		23.333
2. $Y_1 = 100, A_1 = 1$ $Y_2 = 1, A_2 = 50$	1.512		1.485
3. $Y_1 = 100, A_1 = 0.5$ $Y_2 = 5, A_2 = 75$	4.351		4.048

sets of conditions (isotopic masses  $m_a = 32, m_b = 36$ ).

It is of interest to derive a completely general equation for isotope dilution.

Let us consider  $r$  batches, each containing  $n$  isotopes in varying proportions. The notation is as follows:

Atom-% of isotope $a$ in batch $i$	$= A_{ai}$
Number of $a$ atoms in batch $i$	$= N_{ai}$
Total number of atoms in batch $i$	$= N_i$
Total mass of batch $i$	$= Y_i$
Atomic weight of isotope $a$	$= m_a$

The fundamental relations are similar to those expressed in equations (1)–(6):

$$A_{ai} = 100 N_{ai} / N_i, \quad (21)$$

$$\sum_{a=1}^n m_a N_{ai} / L = Y_i, \quad (22)$$

$$\sum_{a=1}^n A_{ai} = 100, \quad (23)$$

$$\sum_{a=1}^n N_{ai} = N_i. \quad (24)$$

Solving these equations as above, one obtains

$$N_i = 100 Y_i L / \sum_{a=1}^n m_a A_{ai}, \quad (25)$$

$$N_{ai} = A_{ai} Y_i L / \sum_{a=1}^n m_a A_{ai}. \quad (26)$$

The atom-% of  $a$  in the final mixture is defined by:

$$A_{af} = 100 \sum_{i=1}^r N_{ai} / \sum_{i=1}^r N_i. \quad (27)$$

Substituting (25) and (26) in (27) gives:

$$A_{af} = \sum_{i=1}^r A_{ai} Y_i R_i / \sum_{i=1}^r Y_i R_i,$$

where

$$R_i = \prod_{k=1}^n \sum_{a=1}^n m_a A_{aki}, \quad (29)$$

where  $\Pi$  as usual denotes the formation of a product. For example, if  $r = 3$ ,  $n = 3$ ,  $R_1 = (m_1 A_{112} + m_2 A_{212} + m_3 A_{312})(m_1 A_{113} + m_2 A_{213} + m_3 A_{313})$ .

The atom-% excess is introduced as before, denoting the normal abundance of  $a$  by  $A_a$ :

$$C_{ai} = A_{ai} - A_a, \quad (30)$$

$$C_{af} = A_{af} - A_a. \quad (31)$$

Introducing (30) and (31) into (28) we obtain:

$$C_{af} = \sum_{i=1}^r C_{ai} Y_i R_i / \sum_{i=1}^r Y_i R_i. \quad (32)$$

As before, a simple case occurs if the atomic weights of all isotopes can be considered approximately equal ( $m_a = m_b = \dots = m$ ). Then (28) and (32) become, respectively:

$$A_{af} = \sum_{i=1}^r A_{ai} Y_i / \sum_{i=1}^r Y_i, \quad (33)$$

$$C_{af} = \sum_{i=1}^r C_{ai} Y_i / \sum_{i=1}^r Y_i. \quad (34)$$

## II. THE EQUATIONS OF ISOTOPE DILUTION FOR COMPOUNDS

The equations to be used for calculations when batches of isotopic compounds, rather than elements, are mixed, can be developed in a strictly analogous manner.

In this case, the notation is modified as follows. For simplicity the case of hydrogen is considered and deuterium (D) is assumed to be the tracer isotope.

	Batch 1	Batch 2
Number of D atoms	$N_{a1}$	$N_{a2}$
Total number of D and H atoms	$N_1$	$N_2$
Atom-% of D	$A_1$	$A_2$
Mass of compound (in g.)	$X_1$	$X_2$
Molecular weight of compound	$M_1$	$M_2$

It should be noted that atom-% D in a compound is defined as 100 (No. of D atoms)/(No. of D atoms + No. of H atoms) rather than the ratio 100 (No. of D atoms)/(Total number of atoms). In addition to the quantities given above, it is further necessary to define  $Z$  = the number of H or D positions per molecule in the particular compound studied; e.g., with palmitic acid,  $Z = 32$ . Then,

$$A_1 = 100N_{a1}/N_1, \quad (35)$$

$$A_2 = 100N_{a2}/N_2, \quad (36)$$

$$N_1 = LZX_1/M_1, \quad (37)$$

$$N_2 = LZX_2/M_2, \quad (38)$$

$$A_f = 100(N_{a1} + N_{a2})/(N_1 + N_2). \quad (39)$$

Substituting from (35)–(38) into (39), we find:

$$A_f = (A_1X_1M_2 + A_2X_2M_1)/(X_1M_2 + X_2M_1). \quad (40)$$

In terms of atom-% excess,

$$C_f = (C_1X_1M_2 + C_2X_2M_1)/(X_1M_2 + X_2M_1). \quad (41)$$

In the general case, we consider any number of batches, containing varying proportions of isotopic material. Suppose further that each batch is homogeneous, i.e., contains one kind of isotopic compound of a definite molecular weight. In a manner similar to equations (35)–(39) the number of atoms of the element (all isotopes) in batch  $i$  is defined as:

$$N_i = LZX_i/M_i. \quad (42)$$

The atom-% of isotope  $a$  of the element in batch  $i$  is:

$$A_{ai} = 100N_{ai}/N_i, \quad (43)$$

where  $L$  is Avogadro's number,  $Z$  is the number of places the element occupies per molecule of the compound,  $M_i$  is the molecular weight of the compound in batch  $i$ ,  $X_i$  is the mass (in g.) of the compound in batch  $i$ , and  $N_{ai}$  is the number of atoms of isotope  $a$  in batch  $i$ .

The atom-% of isotope  $a$  in the final mixture of the batches is:

$$A_{af} = 100 \sum_i N_{ai} / \sum_i N_i. \quad (44)$$

From (42) and (43) we obtain:

$$N_{ai} = LZA_{ai}X_i/100M_i. \quad (45)$$

From (44) and (45):

$$A_{af} = \sum (A_{ai}X_i/M_i) / (\sum X_i/M_i). \quad (46)$$

As before, atom-% excess is defined as the atom-% in a given compound minus the atom-% found in the normal or natural compound. Denoting this by  $C$ , we have:

$$C = A - A_{\text{normal}}. \quad (47)$$

From this it follows that formulae like (46), which are linear and homogeneous in the  $A$ 's, can be transformed to the corresponding formulae for  $C$  by substituting  $C$  for  $A$  (with appropriate subscripts) everywhere. Thus:

$$C_{af} = \sum_i (C_{ai} X_i / M_i) / (\sum_i X_i / M_i). \quad (48)$$

In the usual application, two batches are mixed. Batch 1 is enriched, contains a known amount of the isotopic compound, and its atom-% excess is known as well as the molecular weight. The atom-% excess of the final mixture is obtained experimentally. We wish to know the mass of the (normal) compound present in the unknown batch 2. Applying (48):

$$X_2 = (C_{a1}/C_{af} - 1) X_1 (M_2/M_1), \quad (49)$$

since by the conditions of the experiment  $C_{a2} = 0$  identically.<sup>1</sup>

It has been assumed that the enriched batch 1 is homogeneous, *i.e.*, contains only one kind of enriched molecule of a definite molecular weight. This weight  $M_1$  may be calculated from the experimentally determined atom-% excess of the tracer element. If, in fact, batch 1 were to consist of a mixture of a definite enriched compound and of the normal compound (or of several enriched compounds), we would be averaging in a certain way to obtain  $M_1$  and equation (49). We must see whether this procedure is rigorously correct.

Suppose batch 2 is normal and homogeneous as before, while batch 1 consists of a fraction  $f$  of a "heavy" compound of molecular weight  $M_a$  and a fraction  $(1-f)$  of normal material of weight  $M_b = M_1$ . We apply our general formulae to the final mixture by thinking of it as a mixture of three batches. If the atom-% excess in the enriched fraction of batch 1 is denoted by  $C'_{a1}$ , we get, instead of (49):

$$X_2/X_1 = (C'_{a1}/C_{af} - 1) f M_2/M_a - (1-f). \quad (50)$$

A form more readily comparable with (49) is obtained determining the atom-% excess of batch 1 by treating the batch as a mixture of two batches, which gives:

$$C_{a1} = (C'_{a1} f X_1/M_a) / (f X_1/M_a + (1-f) X_1/M_2). \quad (51)$$

Putting (51) in (50) we get:

$$X_2/X_1 = (C_{a1}/C_{af} - 1) (f M_2/M_a + (1-f)). \quad (52)$$

It remains to be shown that the second factor in parentheses is equal to  $M_2/M_1$ .

<sup>1</sup> It is possible to transform equation (49) into an equation in terms of elements, which could also be derived directly from equation (20) of Section 1. Let  $Y_1$  and  $Y_2$  be the masses of the element in the two batches, and  $m_a$  and  $m_b$  the atomic weights of isotopes  $a$  and  $b$  (tracer and normal). Substituting equation (45) in equations (3) and (4) and taking the ratios of the two expressions, we get, after using (49):  $Y_2/Y_1 = (C_{a1}/C_{af} - 1) R_2/R_1$ , where  $R_1$  and  $R_2$  are defined as before (Equations (11) and (12)).

This is readily accomplished by defining molecular weight in terms of atom-% of the normal and "heavy" isotope, which is the procedure we wish to validate. We have obviously:

$$M_a = A'_{a1}Zm_a/100 + A'_{b1}Zm_b/100 + k, \quad (53)$$

$$M_2 = A_{a2}Zm_a/100 + A_{b2}Zm_b/100 + k, \quad (54)$$

where the  $A'$  refer to the enriched fraction of batch 1, and  $k$  is the molecular weight of the residue of the molecule (part not containing the element under consideration). The  $M_1$  of the mixed batch 1 is accordingly:

$$M_1 = A_{a1}Zm_a/100 + A_{b1}Zm_b/100 + k. \quad (55)$$

Using (53)–(55), keeping in mind that  $A_{a2}$  and  $A_{b2}$  are the  $A_{\text{normal}}$  values, and using (47):

$$(M_1 - M_2)/(M_a - M_2) = C_{a1}/C'_{a1} = (fM_2/M_a)/(fM_2/M_a + (1 - f)), \quad (56)$$

from which:

$$M_2/M_1 = fM_2/M_a + (1 - f), \quad (57)$$

which was to be proved.

It is, therefore, legitimate to apply (49) to isotope dilution with two batches, in which the enriched batch is not homogeneous, but is a mixture of normal and "heavy" compounds. The extension to the more general case is obvious.

### III. THE APPLICATION OF ISOTOPE DILUTION EQUATIONS

We may now consider the manner in which an equation such as (41) is applied to quantitative analysis. A batch of "isotopic" compound of known composition is added to the unknown; the latter contains the same compound with normal isotopic abundance. After thorough mixing, the compound is isolated from the mixture and its isotopic composition is determined. If batch 2 represents the unknown, the only quantity in equation (41) which is not already determined is  $X_2$ ; this is the desired quantity of the compound in the unknown. Solving for  $X_2$  and noting that  $C_2 = 0$  under these conditions:

$$X_2 = (C_1/C_f - 1)X_1(M_2/M_1), \quad (58)$$

which is equivalent to equation (49). When  $M_1 = M_2$  there is obtained the commonly quoted equation:

$$X_2 = (C_1/C_f - 1)X_1. \quad (59)$$

It is of interest to examine the error introduced by neglecting the factor  $M_2/M_1$  which is absent from equation (59). A case in which the effect is not negligible is the analysis for palmitic acid in rat fat cited by Rittenberg and Foster (1). In this instance, the enriched compound



contained 21.5 atom-% excess deuterium. Calculating the molecular weight on this basis, we obtain  $M_1 = 263.25$  whereas  $M_2$  is 256.42. The ratio  $M_2/M_1$  is 0.974, giving a difference of 2.6% between the rigorous and approximate formulas—a difference well outside experimental error.

Equation (55) shows the variation in molecular weight of a tracer compound as a function of the degree of enrichment. The dependence of the molecular weight ratio ( $M_2/M_1$ ) on this factor is clearly shown

TABLE II

*Molecular Weight of Tracer Compounds as a Function of Isotopic Enrichment*

Compound	$M_1$	$Z$	Atom-% D	$M_1$	$M_2/M_1$
Benzene	78.11	6	5	78.41	0.9962
			10	78.70	0.9925
			50	81.08	0.9634
Palmitic acid	256.42	32	5	258.02	0.9938
			10	259.58	0.9878
			50	272.29	0.9417
Glycine	75.07	1	Atom-% $N^{15}$		
			10	75.17	0.9987
			50	75.56	0.9935
			75	75.81	0.9902
Tyrosine	181.19	1	90	75.96	0.9883
			10	181.29	0.9994
			50	181.68	0.9973
			75	181.93	0.9959
			90	182.08	0.9951

in equation (57). In order to illustrate the importance of this effect, we present in Table II a set of model calculations using typical compounds and hypothetical degrees of enrichment with both deuterium and  $N^{15}$  as tracers.

Bearing in mind the degrees of enrichment currently used in tracer experiments, together with the results of Table II, it is apparent that these considerations apply most forcibly to stable isotopes, especially in compounds of low molecular weight. Owing to the greater sensitivity of the detection methods, radioactive isotopes are generally used in

similar types of experiments at extremely low concentrations. Hence, the conditions for the validity of the approximate equation are usually satisfied. However, current trends are toward the use of much more highly enriched radioactive preparations as starting materials. In such cases, it is clear from Table II that the same limitations will be imposed on the use of approximate equations.

### SUMMARY

An exact set of equations for isotopic dilution experiments has been derived. Conditions for application of the approximate equations in general use are discussed. To illustrate the limits of the approximate equation, a set of sample calculations is presented.

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# Action of Cadmium and Thiols on Tissues and Enzymes \*

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## INTRODUCTION

Cadmium fume has been responsible for serious industrial accidents. Its mode of action is thus of practical interest as well as of theoretical importance as a further example of heavy metal effects. Like other heavy metal ions, those of Cd react with tissue proteins to form insoluble metal proteinates (carboxyl groups) and stable metal mercaptides (sulfhydryl groups). This report deals with the action of Cd ions on tissue respiratory systems or the partially purified enzymes, and with the ability of putative therapeutic agents (1) to reverse the Cd action. Thiols are given special attention.

## METHOD

CdCl<sub>2</sub> was added to tissue brei or slices, or to enzyme preparations (at 0°C.) 15 to 30 minutes before readings of oxygen uptake were begun (Warburg technique). The exact amount of tissue brei varied from one experiment to the next, the average moist weight per vessel being 136 mg. for lung (range 108–200), 162 mg. for liver (range 120–210) and 117 mg. for kidney (range 92–154). Moist weight of slices varied, from vessel to vessel, between 113 and 151 mg. for lung, 51 and 74 mg. for kidney, 50 and 67 or 180 and 210, for liver. Substrates were present in final concentrations of 0.03 *M* (Na succinate, Na glycerophosphate) or 0.01 *M* (tyramine), and were tipped either immediately after the equilibration period in the 37.5°C. bath, or after an initial "control" interval of 30 minutes. The pH was maintained at 7.3 with phosphate buffer.

Of the more purified enzyme systems studied, phosphatase was prepared from lung, succinic dehydrogenase from lung or pigeon breast muscle† and choline oxidase and

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† In collaboration with Dr. E. S. G. Barron, who kindly prepared the muscle and liver enzymes.

cytochrome oxidase from liver. The succinic enzyme was extracted from lung by churning the finely ground tissue in the Waring blender for approximately 5 minutes at neutrality, with about 2 volumes each of ice and water as suspension and extraction medium, and subsequently centrifuging at 0°C. Activity was determined for all enzymes except phosphatase by following oxygen uptake. For phosphatase measurement, perfused, autolyzed lung was homogenized, incubated with 0.5% Na glycerophosphate, 0.42% Na barbital buffer at pH 9.5 for about 1 hour, and the trichloroacetic acid filtrate then analyzed for phosphorus (2).

In the fractionation experiments, lungs of dogs exposed to radioactive Cd ( $\text{Cd}^{113}$ , for 30 minutes, average concentration 87.5  $\gamma/\text{L}$ , 3) were well ground (meat grinder and Waring blender), extracted with 0.9% NaCl overnight in the cold, and centrifuged. The residue was again extracted with a volume of 0.9% NaCl equal to the original lung weight. This residue was then extracted with 10% NaCl, and the radioactivity of the subsequent residue and extract was determined (4). The supernatants from the first two saline extractions were combined, adjusted to pH 5 with acetic acid and centrifuged. The resulting supernatant, representing the albumin fraction, was evaporated, ashed and read. The centrifugate, taken up at pH 8, was dropped into boiling acetone, then centrifuged. The acetone-soluble lipid fraction and the acetone-insoluble globulin fraction were then ashed and read.

To determine the effectiveness of thiols in reversing Cd inhibition of tissue respiration or enzyme activity, the test material was added to the Cd-treated system at the end of the "period of contact" (15–30 minutes, see above), or was tipped in from an onset during the course of the experiment. Final thiol concentrations ranged from 0.5 to 200 times the molarity of the Cd present, but ultimate evaluations of thiol effectiveness are based on experiments involving thiols and Cd of equal molarity, mainly  $10^{-4} M$ . When  $10^{-3} M$  or higher, the thiols autoxidize so rapidly and the uptake is of such magnitude (as high as 120 mm.<sup>3</sup> O<sub>2</sub> for  $5 \times 10^{-3} M$  BAL; theoretical 112 mm.<sup>3</sup>), that results are not readily interpretable. Concentrations of Cd and thiol best suited for a series of comparative experiments were determined by first ascertaining the Cd just necessary to give 100% inhibition, then selecting an amount of thiol (using BAL as the standard) providing appreciable but incomplete reversal. The thiols used include a group of NDR compounds (6) with names and reference code numbers as follows: BAL or NDR 133, 1,2-dithioglycerol; 131, 1,3-dithioglycerol; 121, ethanedithiol; 132, propanedithiol-1,3; 134, propanedithiol-1,2; 142, propanetrithiol-1,2,3; 230, 2,3-dimercaptopropyl acetate; 293, 2,3-dimercaptopropyl ethyl ether; 400, N-(2,3-dimercaptopropyl)-carbamate; also glutathione, and a series of compounds related to BAL, prepared according to Kharasch (7), KD2, KD8 and KD9; BAL-ethanolamine-, sarcosine- and glycine-methylene mercaptid, respectively.

## RESULTS

### *a. Action of Cd on Tissues and Enzymes*

The basal respiration of lung brei is fairly sensitive to added Cd (up to 35% inhibition with  $10^{-4} M$   $\text{CdCl}_2$ , 50% with  $10^{-3} M$ ; see Table I), but succinic dehydrogenase is the most sensitive of all lung enzyme systems investigated. Virtually complete inhibition is achieved

by final concentrations of Cd as low as  $10^{-4}$  M. The sulfhydryl group is an essential part of the succinic enzyme and injury might be attributed to a combination between the metal and the  $-SH$  group. However, tyramine oxidase, also a "sulfhydryl enzyme," is fully as active in lung brei exposed to  $10^{-3}$  M Cd as in control brei. Glycerophosphate

TABLE I  
*Cd Inhibition of Tissue Respiration*  
*A. Brei*

Per cent Inhibition CdCl <sub>2</sub>	Basal			Extra Respiration Induced by:					
				Succinate			Ty- ra- mine	Glycero- phos- phate	Aden- ylic Acid
	Lung	Liver	Kidney	Lung	Liver	Kidney	Lung	Lung	Lung
$10^{-3}$ M	46 (40-50, 6)*	40 (35-50, 3)	47 (35-55, 3)	100 (2)	100 (3)	100 (3)	0 (2)	0 (1)	0 (1)
$10^{-4}$ M	24 ( 0-35, 4)		0-30 (2)	90 (52-100, 5)	30 (1)	100 (3)	0 (1)	0 (1)	0 (1)
$10^{-5}$ M			15 (1)		0 (1)	0 (1)	0 (1)		

\* Bracketed figures give range of inhibition, followed by number of experiments averaged.

*B. Slices*

Per cent Inhibition CdCl <sub>2</sub>	First Hour			Second Hour			Third Hour		
	Lung*	Liver**	Kidney*	Lung	Liver	Kidney	Lung	Liver	Kidney
$10^{-3}$ M	0	0	47	22	25	74	—	20-40	85
$10^{-4}$ M	0	0	24	19	0-22	53	—	0-25	54

\* One experiment each.

\*\* Two experiments.

and adenylic acid which, like tyramine, increase lung brei oxygen uptake two- or three-fold (5), are also metabolized as readily in the presence of  $10^{-3}$  M Cd as in its absence. The respiration of lung slices is depressed approximately 20% by  $10^{-4}$  M Cd, but only in the second hour, and ten times this amount has but little additional effect. Difficulties of penetration by the metal ion into the slice may well account for this difference in response of the 2 types of tissue preparations.

Liver and kidney breis, when treated with Cd *in vitro*, react about the same as does lung brei (see Table I). Liver slices are injured to about the same extent as lung slices; kidney slices are much more seriously affected.

The more purified phosphatase and cytochrome oxidase are relatively uninjured by Cd (see Table II), while succinic dehydrogenase is still most sensitive.

TABLE II  
*Cd Inhibition of Enzymes*

$\frac{\text{Per cent Inhibition}}{\text{CdCl}_2}$	Phosphatase	Succinic Dehydrogenase		Choline Oxidase	Cytochrome Oxidase
		Lung	Muscle		
$10^{-3} M$	25	100	100		
$5 \times 10^{-4} M$	10				
$10^{-4} M$	0	99 (91-100, 4)		58*	0*
$5 \times 10^{-5} M$			100	18*	
$10^{-5} M$		65	70		
$10^{-6} M$		45	12		

\* Figures kindly supplied by Dr. E. S. G. Barron.

In an attempt to tag the tissue component with which Cd combines, the lungs of three dogs exposed to radioactive Cd were fractionated. The average concentration in each fraction, expressed as  $\gamma$  Cd/g. dry weight, is: albumin, 120; globulin, 60; lipid, 4.5; insoluble in 10% NaCl, 33; soluble in 10% NaCl, 4.

#### *b. Antagonism of Cd and Thiols; Thiol Inhibitions*

Just as the thiols exert a toxic effect when administered to animals (8), so do they inhibit respiration of tissues and activity of partially purified enzymes. The NDR compounds may be listed in order of decreasing "*in vitro* toxicity" about as follows: 142; 293, 230; 131 and 400; BAL, 134, 121, and 132. The first 3 are definitely more toxic than BAL, 142 decidedly so. The rest approximate each other closely, as may be seen in Table III.

In this table is shown also the degree of protection given by Cd previously added. In every instance the Cd itself inhibits more than does the thiol, and only when the two together erase all inhibitory effect are their effects on each other clear-cut. Although the degrees of "inhibition prevention" (by Cd) and "inhibition reversal" (by thiol) may not always be properly apportioned, the following convention has

TABLE III  
*Thiol Inhibitions and Their Prevention by Cd*

Symbol	Concen., moles	CdCl <sub>2</sub> added, moles	Purified Succinic dehydrogenase		Succinic dehydr. of:		Liver slice basal resp
			Lung	Muscle	Lung	Kidney	
BAL	$5 \times 10^{-3}$	—			100† (10)*	95	40
	"	$10^{-3}$					
	"	$10^{-4}$				(10)	
	$3 \times 10^{-3}$	—		0			
	$5 \times 10^{-4}$	—			30		
	"	$10^{-4}$			(85)		
	$2 \times 10^{-4}$	—			20		
	"	$10^{-4}$			(100)		
	$10^{-4}$	—	5		3		
	"	$10^{-4}$	(100)				
131	$5 \times 10^{-4}$	—			50		
	$5 \times 10^{-4}$	—			30		
	"	$10^{-4}$			(100)		
	$10^{-4}$	—	7		35		
	"	$10^{-4}$	(100)		(50)		
132	$5 \times 10^{-4}$	—			10		
	"	$10^{-4}$			(100)		
	$10^{-4}$	—	5		0		
	"	$10^{-4}$	(100)				
134	$5 \times 10^{-4}$	—			45		
	"	$10^{-4}$			(75)		
	$10^{-4}$	—	10		10		
	"	$10^{-4}$	(100)		(100)		
121	$10^{-4}$	—	0		7		
	"	$10^{-4}$			0		
142	$10^{-4}$	—	20		65		
	"	$10^{-4}$	(0)		(30)		
230	$10^{-4}$	—	15		20		
	"	$10^{-4}$	(100)		(100)		
293	$10^{-4}$	—	15		30		
	"	$10^{-4}$	(100)		(50)		
400	$10^{-4}$	—	0		15		
	"	$10^{-4}$			0		
GSH	$5 \times 10^{-3}$	—				30	
KD2	$5 \times 10^{-4}$	—		70			

\* Bracketed figures give the *per cent* reversal by Cd of the thiol inhibition.

† 35% in one experiment.

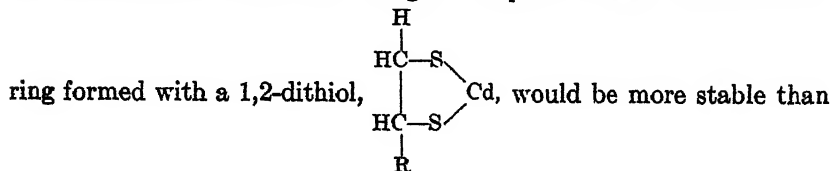


been adhered to in expressing the results: If the depression of activity in the presence of both Cd and thiol is greater than that with thiol alone, but less than with Cd alone, then it is assumed that the thiol effect has been wholly prevented, and that the difference between inhibition with Cd alone and that with Cd plus thiol represents the extent of reversal of the Cd action achieved by the thiol. If the depression is greatest with Cd, less severe with thiol, but mildest with both, then reversal of Cd injury is considered complete, with partial prevention of thiol damage. Though this latter conclusion is not necessarily valid, it was decided upon since, in one experiment involving BAL and 131, of like concentration but 5 times the Cd present, the BAL itself inhibited, 131 did not. 131 completely reversed the Cd inhibition, BAL left a small depression. It seems reasonable to assume that both thiols fully counteracted the Cd, but that BAL, in addition, produced its own slight inhibition.

It is interesting to note that  $10^{-3}$  M Cd inhibits the autoxidation of BAL and of 131 ( $5 \times 10^{-3}$  M) by 35–85%. This observation merely supplements analytic evidence of Cd-thiol combination, such as the lowered iodine titration of  $-SH$  following treatment of the thiol with Cd.

### *c. Antagonism of Cd and Thiols; Thiol Reversal of Cd Inhibition*

Early toxicity studies (8), borne out by work with radioactive Cd (4), showed CdS to be many times less toxic than the chloride or oxide—presumably because of its very low solubility. Inhaled  $H_2S$ , directly reaching the toxic agent at the site of injury, might thus decrease injury by inactivating and fixing inhaled  $CdCl_2$  or  $CdO$ . The lower aliphatic mercaptans, on injection, could act similarly and, in addition, are much less toxic than  $H_2S$ . It might be expected that the 5-membered



the corresponding  $R-S-Cd-S-R$  obtained with two molecules of monothiol. BAL or  $H_2S$  can, in fact, each capture  $Cd^{++}$  from combination with the other; while  $Cd^{++}$  is fully removed by BAL from  $CdO$  (10). There is, however, no evidence that  $Cd^{++}$  reacts specifically with

TABLE IV  
Inhibition and its Reversal by Thiols

		Per cent Inhibition of Enzyme or Tissue System											
		CdCl <sub>2</sub> , Moles	Pure Succinic Enzyme		Lung Respiration			Kidney Resp		Liver Resp			Pure Choline Oxidase
			Muscle	Lung	Brei		Slices	Brei		Brei		Slices	
					Basal	Succinate		Basal	Basal	Succinate	Basal		Succinate
		10 <sup>-3</sup>			44	64	22	47	100	42	96	25	
		10 <sup>-4</sup>		100*		96†	19		100		30	15	60
		5×10 <sup>-5</sup>	100										20
Thiol		Per cent Reversal by Thiol of Cd Inhibition											
Sym-bol	Conc , Moles												
BAL	5×10 <sup>-3</sup>	10 <sup>-3</sup>			100	100		100	100	70		0	
	3×10 <sup>-3</sup>	10 <sup>-4</sup>				67						0	
	5×10 <sup>-4</sup>	5×10 <sup>-5</sup>	100										
	"	10 <sup>-4</sup>				100							
	2×10 <sup>-4</sup>	"				83							
	10 <sup>-4</sup>	"		60		63							
	5×10 <sup>-5</sup>	"				19							
131	5×10 <sup>-3</sup>	10 <sup>-3</sup>			100	100							
	10 <sup>-4</sup>	10 <sup>-4</sup>		45		100							
132	5×10 <sup>-4</sup>	10 <sup>-4</sup>				100							
	10 <sup>-4</sup>	"		27		31							
134	10 <sup>-4</sup>	10 <sup>-4</sup>		53		41							
121	"	"		75		100							
142	"	"		100		100							
230	"	"		48		100							
293	"	"		75		100							
400	"	"		69		100							
KD9	"	"		0									
KD2	5×10 <sup>-4</sup>	5×10 <sup>-5</sup>	0										
GSH	10 <sup>-3</sup>	10 <sup>-3</sup>					0					0	
	"	10 <sup>-1</sup>					100					0	50
	"	5×10 <sup>-5</sup>											100
	5×10 <sup>-3</sup>	10 <sup>-3</sup>						100	0	60	40	0	
	"	10 <sup>-4</sup>							15			0	
	3×10 <sup>-3</sup>	10 <sup>-3</sup>								55	12		
	"	10 <sup>-1</sup>									78		
	7×10 <sup>-4</sup>	5×10 <sup>-5</sup>	100										

+ 94% in one slightly divergent experiment.

† 55% and 52% in 2 divergent experiments.

a natural 1,2-dithiol, nor is a dithiol rather than monothiol required for successful competition and therapy—as in the case of arsenic (11).

Monothiols, dithiols and a trithiol (NDR 142) showed increasing efficacy against  $\text{Cd}^{++}$ , and also increasing toxicity, molecule for molecule. With  $10^{-4} M$  Cd as the inhibitory agent and succinic dehydrogenase of lung brei as the system, BAL in the following quantities reversed the inhibition as indicated:  $5 \times 10^{-4} M$ , 100%;  $2 \times 10^{-4} M$ , 83%;  $10^{-4} M$ , 63%;  $5 \times 10^{-5} M$ , 19% (see Table IV). Therefore,  $10^{-4} M$  was the thiol concentration selected for the series of comparative experiments summarized in Table IV. It is clear that 142, the very noxious trithiol, is most effective in counteracting Cd. 293, also more harmful than BAL, is likewise a somewhat better reversing agent. 230 and 131, the former a little more injurious than BAL, about equal BAL's reversal. 134, 121, 400, and 132 are perhaps slightly less effective than BAL. Differences between the compounds in these last 2 groups are small, however, and their respective positions shift from one system to another. In general, it may be said that the most effective thiols are also the most inhibitory in their own right, so that none of the compounds here tested suggests itself as a possible therapeutic rival of BAL.

### SUMMARY

Cd reacts with tissue proteins, causing alteration in structure or decrease in solubility sufficient to impair enzyme activity, thereby making cellular function impossible. Combination between Cd and —SH groups is readily demonstrable, but neither this factor nor the formation of insoluble Cd proteinate can completely explain Cd toxicity to the lung. Some —SH-containing enzymes are not affected by Cd in concentrations that completely inhibit others, nor do some other heavy metals share the drastic effects of Cd (8). Specific steric features of protein structures may help account for both of these findings.

Thiols are remarkably effective in reversing the *in vitro* Cd inhibitions considered here, but the mechanism of this reversal is not immediately apparent. Therapeutic BAL does not alter the content of Cd in the lung nor its rate of departure (4), and animals receiving BAL prophylactically have a higher lung Cd content than do controls, and die sooner (4, 8). Hence the therapeutic effect cannot be due simply to immobilization of Cd. Another possibility is that therapy converts a  $\text{Cd}(\text{protein})_2$  compound to a less toxic BAL-Cd-protein. The finding

(9), that insoluble Cd BAL can be converted into soluble Cd(BAL)<sub>2</sub> with excess of the dithiol, is pertinent in this connection.

Of the various lung, liver, kidney and muscle enzyme systems investigated, succinic dehydrogenase is most readily inhibited by Cd. Choline oxidase is less sensitive, while alkaline phosphatase and cytochrome oxidase are but slightly, if at all, affected. Utilization of tyramine, glycerophosphate and adenylic acid by lung brei is unimpaired even by  $10^{-3}$  M Cd. Mono-, di- and trithiols, while exerting a toxic action themselves, do reverse the Cd injury. Of the thiols tested, BAL, 1,2-dithioglycerol, is itself least toxic and best satisfies the requirements for counteracting Cd inhibition. That these conclusions based on *in vitro* experiments also apply to the living animals is evident from the extensive *in vivo* investigations of Gerard *et al.* (8).

#### ACKNOWLEDGMENT

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# Protein Utilization by the Adult Rat: the Lysine Requirement<sup>1</sup>

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The first clear indication of which the writer is aware that protein utilization in the adult rat differs from that in the growing rat was presented by Osborne and Mendel (1) in 1916. In as careful a protein comparison as can be found in the literature, they showed that growing rats on the same caloric intake made equal gains in body weight when their protein supply was derived from diets containing 9% of lactalbumin, 12% of casein or 15% of edestin. In these tests 32.0 g. of lactalbumin, 49.2 g. of casein and 59.9 g. of edestin were equivalent in growth-promoting potency. However, in the maintenance of adult rats, the requirements of these 3 proteins expressed in mg./week/g. of rat were 12.5 for lactalbumin, 16.4 for casein and 15.0 for edestin. It will be noted that the relative values of casein and edestin have changed in the 2 series of experiments: for growth, casein is the better by a considerable margin, while for the maintenance of the adult, edestin is somewhat better than casein. The lysine deficiency of edestin, which limits its value for growth (2), is no handicap in its value in adult nutrition.

Again, in 1919, in their classical paper (3) on the nutritive value of the wheat kernel and its milling products, the same investigators showed that the proteins of wheat endosperm, even at a level of 15% in the diet, supported very little growth in the rat, much less than the proteins of whole wheat, although for the maintenance of the adult rat they were very nearly, if not quite the same in value: 23 to 26 mg. of endosperm proteins were required for this purpose/g. of rat/week, against 20–23 mg. of whole wheat proteins. Again, a lysine deficiency, in the endosperm proteins (4) this time, was not a serious handicap in maintaining the nitrogenous integrity of the tissues in adult life.

The small, if any, role that the amino acid lysine plays in the nutrition of the adult rat was indicated earlier by these pioneer investigators in modern nutrition (5) in their demonstration that a diet, containing zein+tryptophan, devoid of any considerable source of lysine, can maintain rats at constant body weight and apparently in good condition, for long periods of time.

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<sup>1</sup> This paper was presented before the Division of Agricultural and Food Chemistry at the Meeting of the American Chemical Society in Chicago, September 9–13, 1946. Some of the experimental data reported in this paper was obtained with the aid of funds contributed by General Mills, Inc., of Minneapolis, Minnesota. The author is grateful for this assistance.

It is true that these investigations of Osborne and Mendel, minimizing the importance of lysine in the nutrition of the adult rat, are marred somewhat in their obvious significance by the presence in many of their test diets of 28% of a milk preparation said to be protein-free, but nevertheless containing a small amount of nitrogen, about 0.7% (6).

About 6 years ago, Burroughs and others (7) made a study of the amino acids required by the adult rat for the replacement of endogenous losses of nitrogen. This investigation showed that nitrogen equilibrium can be maintained in mature rats on diets lacking some of the amino acids shown by Rose (8) to be essential for growth, specifically histidine, leucine, phenylalanine, arginine and lysine. The evidence for the dispensability of lysine was not entirely satisfactory in all tests, but certainly its absence from the diet was compatible with at most only small losses of nitrogen, well within the experimental error of a metabolism experiment. The failure of Neuberger and Webster (9) to confirm the dispensability of lysine by the adult rat rests upon their failure to maintain the body weights of mature rats on lysine-deficient diets consumed *ad libitum*. Whether the loss of weight was primarily due to a caloric deficiency or to a lysine deficiency cannot be decided from the evidence presented. It should be noted that Burroughs assured an adequate caloric intake for all of his experimental diets by the expedient of tube feeding. Under these conditions body weight could be maintained, as well as approximate nitrogen equilibrium, for periods of 6-8 days on lysine-deficient diets. The same remarks apply to the failure of Albanese and Frankston (10) to maintain mature rats at constant body weight on histidine-deficient diets.

TABLE I

*The Utilization of Food Proteins by the Growing Rat, 10% Level of Protein in Diets*

Protein source	Protein utilization		
	True digestibility	Biological value	Net utilization
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Egg, whole	100	94	94
Beef round	99	76	75
Milk, dried defatted	93	84	80
Soy flour	96	75	72
Rolled oats	93	66	61
White flour	100	52	52
Whole wheat	91	67	61
Cereal mixture (oat, wheat)	92	65	60
Beef + white flour <sup>1</sup>	100	73	73

<sup>1</sup> 2 Parts of flour N to 1 part of beef N (16).

In recent years, our laboratory has carried out studies on the digestibility and biological value of many food proteins on both growing and mature rats, using either the nitrogen-balance technic as originally

proposed (11) but modified in later publications (12), or an adaptation to adult rats of the nitrogen-balance technic of Melnick and Cowgill (13) involving the computation of regression equations of nitrogen balance on nitrogen intake (14). Some of these results on growing rats are presented in Table I.

It will be noted that, for the growing rat, there is a clear distinction between the animal foods and the cereal foods in the metabolic utilization of nitrogen as measured by the biological value. In digestive utilization the distinction is not as clear when account is taken of the metabolic nitrogen appearing in the feces. Furthermore, there is a marked supplementary relation between the proteins of beef and those of white flour, since the biological value of the mixtures is  $73\sigma_C$ , a value almost as high as that for beef alone.

In Table II are assembled similar results for adult rats. In the experiments yielding these results, the level of protein in the diets was

TABLE II  
*The Utilization of Food Protein by the Adult Rat, 4-5% Protein in Diets*

Protein source	Protein utilization		
	True digestibility	Biological value	Net utilization
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Egg, whole <sup>1</sup>	100	100	100
Beef round	99	73	72
Milk, dried defatted <sup>1</sup>	95	96	91
Soy flour <sup>1</sup>	90	54	49
Rolled oats	95	82	78
White flour as bread	95	65	62
Whole wheat	93	74	69
Cereal mixture (oat, corn, rye)	96	79	75
Beef+white bread <sup>2</sup>	100	70	70

<sup>1</sup> Taken from Doctor's Thesis of Mildred Bricker, University of Illinois, 1945.

<sup>2</sup> In the proportion of beef N 2.4 to white flour N 1.0.

reduced to 4 or 5% in order to induce negative nitrogen balances with the assurance that the protein supply was not excessive. In comparison with the data of Table I on growing rats, the difference in metabolic utilization of the nitrogen of animal foods and of cereal foods has narrowed. In fact, the nitrogen of beef round is less well utilized in metabolism than that of rolled oats, and this difference is statistically



significant. The biological values of the nitrogen of whole wheat and of the cereal mixture (consisting largely of oats) are as high as, or higher than, that for beef. Even the biological value for white bread nitrogen is not greatly inferior to that for beef nitrogen, and when the two are combined no evidence of a supplementary effect appears. This situation contrasts with the same situation in the protein nutrition of growing rats.

The facts just discussed with reference to protein utilization in the adult rat as compared with the immature rat suggest that the lysine requirement of the rat during the period of growth either disappears entirely when growth is complete, or assumes an inconspicuous role in comparison with the requirements for the other amino acids. Cereal proteins are known to be generally deficient in lysine (15) and animal proteins, generally somewhat deficient in cystine and methionine, are, as a class, rich in lysine. If the lysine requirement disappears on the completion of growth, then the cereal proteins should prove to be much more valuable for the rat in adult nutrition as compared with animal proteins than in adolescent nutrition. Furthermore, the marked supplementary relations known to exist in the growing rat between beef proteins and white flour proteins (16) may not be evident in the mature animal, especially since methionine is the second limiting amino acid for white flour proteins (15). These *sequelae* of the disappearance of the lysine requirement in adult rodent nutrition are revealed by the data of Table II.

To secure more direct evidence on this question, 4 mature male rats were placed upon a white flour diet, first without and then with a supplement of lysine. Four other mature male rats were placed upon a beef diet, first without and then with a supplement of cystine. In the comparative periods the food consumption of the rats was the same, and the nitrogen intakes were approximately the same also. The nitrogen output in the urine was then determined for each rat during each period, as being the best indication of supplementary effects. The pertinent data have been summarized in Table III.

It will be noted from the Table that, whereas a cystine supplement decreased the wastage of beef nitrogen in the urine for all 4 rats, a lysine supplement to white flour protein gave an indecisive result: the urinary nitrogen decreased in 2 cases and increased in the other 2. The data are thus consistent with the conclusion supported by previous experiments that, in the nutrition of the adult rat, lysine is

either a dispensable dietary component, or the requirement for it is very small indeed in comparison with that for other amino acids.

On the other hand, the cystine requirement of the growing rat persists into adult life, and perhaps this is why proteins of the soybean, deficient in cystine and even more so in methionine (15), are distinctly less well utilized in adult rodent nutrition than in adolescent nutrition (compare Tables I and II).

TABLE III  
*Amino Acid Supplementation of Beef and Flour Proteins in the  
Adult Rat. 4-5% Protein in Diets*

Rat number	Food consumed daily <i>g.</i>	Without amino acid supplement		With amino acid supplement		Difference in urinary N <i>mg</i>
		N intake <i>mg.</i>	Urinary N <i>mg.</i>	N intake <i>mg.</i>	Urinary N <i>mg.</i>	
Beef, with and without cystine <sup>1</sup>						
1	10	71.9	58.2	71.8	39.9	-18.3
3	10	71.9	55.4	71.8	38.9	-16.5
5	10	71.9	61.8	71.8	53.9	-7.9
9	10	71.9	59.0	71.8	44.1	-14.9
White bread, with and without lysine <sup>2</sup>						
4	10	67.6	64.9	75.8	54.7	-10.2
6	10	67.6	58.1	75.8	63.1	+5.0
8	10	67.6	64.7	75.8	61.8	-2.9
10	10	67.6	60.0	75.8	64.0	+4.0

<sup>1</sup> The supplemented ration contained 0.12% *L*-cystine as an *addendum*.

<sup>2</sup> The supplemented ration contained 0.28% *L*-lysine monohydrochloride as an *addendum*.

The conclusion supported by the above observations that lysine may not be required at all by the adult rat relates only to the maintenance of nitrogen equilibrium. As Holt and Albanese (17) have pointed out, the attainment of nitrogen equilibrium and its maintenance over short periods of time on an amino acid mixture does not necessarily constitute final evidence of its complete nutritive adequacy. A negative nitrogen balance in some vital but quantitatively insignificant tissue may be entirely obscured in the nitrogen balance of the

entire organism. In the instance under discussion, no conclusion can be drawn as to the dispensability (or otherwise) of lysine for reproduction in the adult rat. Osborne and Mendel (20) reported the birth of a litter of 4 by a female rat that had subsisted for 178 days on a diet containing 18% of gliadin as the sole test protein. In the immature female rat, Pearson (18) has shown that a lysine-deficient diet suppresses not only growth but also the estrous cycle. The latter effect may persist into adult life, or it may be merely an effect of any type of undernutrition, such as a restricted caloric intake (19).

The insignificant role that lysine plays in the protein anabolism of the adult rat contrasts sharply with the prominent place of lysine in the protein nutrition of adult man (8, 17). Except for the dispensability of histidine and arginine, the amino acids required by the adult human for the maintenance of nitrogen equilibrium seem to be identical with those required by the growing rat. The adult rat, however, does not need for this purpose, not only lysine, but also histidine, leucine, phenylalanine (if tyrosine is present in the diet) and arginine (7).

This difference between the rat and man in the dietary amino acids required for mere maintenance of the *status quo* of the tissues in adult life and those required for maintenance plus growth in the immature animal, does not seem to be an expression of differences in the amino acids needed to replace endogenous losses and those needed for protein synthesis. While endogenous losses of nitrogen seem to relate largely to the catabolism of non-protein nitrogenous constituents of the tissues (21), they also must involve protein catabolism as well. The continuous destruction of the red cells of the blood and their contained hemoglobin is an illustration of a type of catabolism that is quite possibly occurring in many tissues of the body. Furthermore, the similarity in the end-products of the minimum endogenous nitrogen catabolism and its close relationship to the basal metabolism of energy in many species of animals (26) affords circumstantial evidence that the amino acids involved in this catabolism are the same in different species.

If the amino acids involved in the maintenance metabolism may be assumed to be the same for rat and for man, the difference in dietary requirements of amino acids can be accounted for, it would seem, on the assumption that some, or all, of the amino acids essential for growth in the rat are synthesized in the tissues, but only at rates that are quite inadequate to cover the requirements for growth. This is known to be

true for arginine in the rat (8) and for both arginine and glycine in the chick (25). When growth is completed, these synthetic reactions may or may not be capable of supplying the amino acid requirements of maintenance, or at least some of them.

Whether they do or do not may depend upon the intensity of the synthetic reactions of growth per unit weight of metabolizing tissue. In a rapidly growing animal, such as the rat, this intensity is great. When growth is completed, there is a large reduction in the need for synthesis; hence, conceivably the residual capacity to synthesize cellular components, including the "essential amino acids," may be adequate to cover the requirements for some of them, such as arginine, histidine, lysine, leucine and phenylalanine, for the replacement of endogenous losses. In such cases, the dietary requirement disappears.

In the case of a slow-growing animal, such as man, the transition from adolescence to maturity may involve such a slight reduction in the intensity of anabolism per unit weight of tissue that reactions involving the synthesis of "essential amino acids" may be appreciably no more able to cover the requirements for maintenance than they were the requirements for growth. In such case, the amino acids required in the diet for the adult may be much the same as those required for the child.

The great difference in the growth rate of the rat and of the child has been noted by Brody (22), measuring the growth rate by  $[dw/dt \times 100 \div W]$ . During fetal life, the rate for the rat is about 35 times that for the human; comparing the juvenile period of the child with as nearly comparable a period for the rat as possible, the growth rates are in the proportion of 100:1. During the period of self-inhibiting growth, computations made by the author from the data of Smith and Bing (23) for the rat, and of Meredith (24) for the child, indicate ratios ranging from 41:1 at a time when half the mature weight is reached, to 1:1 when maturity is nearly reached.

### CONCLUSIONS

A lysine deficiency in food proteins, as in cereal foods, does not limit their ability to maintain nitrogen equilibrium in the adult rat. Such foods are not improved in their protein value by combination with lysine-rich proteins nor by additions of lysine itself.

On this and other grounds it is concluded that lysine is entirely dispensable in adult rodent nutrition, or is required in inconspicuous

proportions for the maintenance of nitrogen equilibrium. This conclusion is applied only to the sexually inactive rat.

A theory is proposed to account for the fact that the adult rat requires fewer amino acids for the maintenance of nitrogen equilibrium than does the adult human.

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# Studies in Protein Synthesis *in vitro*. I. On the Synthesis of Labeled Cystine ( $S^{35}$ ) and its Attempted Use as a Tool in the Study of Protein Synthesis

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## INTRODUCTION

### *Protein Synthesis*

Due to the great importance of the biological synthesis of protein, many workers have sought suitable *in vitro* systems in which it could be studied. The first efforts aimed at showing that synthesis could result from the reversal of enzymic hydrolysis by decreasing the concentration of water. This work has been reviewed by Borsook and Wasteneys (1). Protein-like materials (plastein) were obtained but there is considerable doubt whether there was ever any significant increase in peptide bonds (2). In some work plasteins were formed by linking protein split products with disulfide bridges (3). On this account, and also because the conditions depart so widely from those likely to exist in protoplasm, other methods of investigation have been sought.

Bergmann and coworkers (4) have made use of product insolubility to displace equilibria in favor of peptide bond formation. Thus, they have succeeded in synthesizing many insoluble peptides by bringing their components together in the presence of the appropriate proteolytic enzymes.

Although various arguments have been advanced purporting to show that such methods of displacing equilibria could account for protein synthesis in the organism yet the demonstration of peptide bond synthesis by such means does not even imply that the equilibrium is thus displaced in favor of synthesis in living systems.

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It is now known that many of the synthetic (endergonic) processes which take place in the organism go only by virtue of more or less direct chemical coupling with energy-yielding reactions (5, 6, 7, 8). Consequently, to investigate protein synthesis it is necessary to adopt some method which will permit energy-yielding reactions to proceed. Such methods are available in the tissue slice and bolstered homogenate techniques, but these techniques as ordinarily employed are useless because there is an increase in non-protein nitrogen and hence a decrease in protein when slices are incubated under the most favorable conditions (9). However, it should be possible by employing labeled amino acids to demonstrate incorporation of amino acid into protein (protein synthesis) even though there may be a net decrease in protein-bound amino acid. This has in effect already been shown in intact animals fed labeled acids (10). The amino acids are readily incorporated into proteins *in vivo* although the animals are not growing, that is, the incorporation is not due to net increase in protein. The present paper represents an attempt to use cystine labeled with radioactive sulfur to show synthesis in liver preparations.

Labeled cystine was added to the slices or homogenates or heated homogenates in Krebs' saline and the preparations were incubated in 95% O<sub>2</sub>-5% C<sup>18</sup>O<sub>2</sub> for 1.5-2 hours. Then the preparations were homogenized, the pH adjusted to about 6, the protein precipitated, centrifuged down and washed well. The total sulfur in the protein precipitate was converted to barium sulfate and radioactivity measurements made to determine the incorporation of the label into the proteins.

It was all too easy to show the uptake of cystine by slice proteins and even more so by the proteins in homogenates. The uptake was so great that the conclusion became inevitable that it must be due to the binding of cystine by linkages other than peptide. This proved to be the case because most of the cystine was easily removed by reduction under controlled conditions.

*The results show that it is necessary to proceed with the utmost caution in any work with cystine when the amino acid is used to investigate protein metabolism.*

#### *Cystine Synthesis*

Various methods have been employed for the synthesis of cystine. The earlier methods of Erlenmeyer and of Fischer (11, 12) depend on the conversion of serine to cystine. More recently Bohme's method of making mercaptochlorides has permitted syntheses by variations of

the malonic ester method (13). However, none of the described methods is suited to the preparation of cystine containing labeled sulfur.

The *S*-benzyl derivative of cysteine is readily prepared by acid hydrolysis of the product resulting from the condensation of sodium benzyl mercaptide with  $\alpha$ -benzamido- $\beta$ -chloropropionate. *S*-benzylcysteine is readily converted to cyst(e)ine, thus providing a method in which the yield, based on the sulfur used, is good. It should be possible to prepare *d*- or *l*-cystine by starting with the appropriate optically active chloro compound.

## EXPERIMENTAL

### *Synthesis of Cystine*

Ethyl  $\alpha$ -amino- $\beta$ -chloropropionate hydrochloride was prepared from *dl*-serine ester hydrochloride by the method of Fischer and Raske (14). The product was benzoylated by the method of Karrer and coworkers (15). The reaction between the benzamido compound (10 mM), benzyl mercaptan (5.35 mM) (16) and potassium (6 mM) in 6 ml. ethyl alcohol (absolute) was conducted by heating for 30 min. at 65–75°C. To the reaction mixture was added 125 ml. 6 *N* HCl. After refluxing for 19 hours hydrolysis was complete and the solution was virtually clear. The benzoic acid was separated by filtering it from the ice-cold hydrolyzate. Any *S*-benzylcysteine precipitated with the benzoic acid was washed out with several small portions of ice water. The filtrate and washes were taken down to dryness at reduced pressure, the residue dissolved in water, and the *S*-benzylcysteine precipitated by adjusting to pH 6 with ammonia. After chilling the product was filtered off and washed with several portions of alcohol and ether. Yield: 0.90 g. or 80% on the basis of the benzyl mercaptan used. For analysis the compound was recrystallized from boiling water.

Calculated *S* = 15.19

Found *S* = 15.48, 15.42

*N* = 6.64

*N* = 6.55, 6.63, 6.77

The *S*-benzylcysteine was converted to cystine by the method used by Wood and du Vigneaud (13).

### *Incubation Experiments*

The tissue preparations were all made from the livers of 60-day old rats most of which were fasted 24 hours before the experiment.

Tissue slices about 0.5 mm. thick were prepared from the livers. Homogenates were made in an all glass apparatus as described by Potter and Elvehjem (17).

The incubation of all preparations was carried out at 37–38°C. in Krebs' saline (18) slightly modified by substituting Na<sub>2</sub>HPO<sub>4</sub> for some of the KH<sub>2</sub>PO<sub>4</sub>, so that it was at pH 7.4 when equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Incubation was done in 125 ml. glass-stoppered Erlenmeyer flasks with agitation as in a conventional Warburg apparatus. The labeled cystine used was dissolved in 0.1 *N* HCl and neutralized just before adding to the medium. No precipitation was observed.



After the incubation period of 1.5–2 hours the preparations were transferred to centrifuge tubes (slices were homogenized), diluted to about 50 ml., adjusted to about pH 5.6 and the proteins precipitated by heating. The precipitated materials were washed with acetate buffer (pH 5.6). In some experiments the deproteinized media and washes were made to a convenient volume (250 ml.) and aliquots removed for determination of labeled sulfate. The protein sulfur was converted to sulfate by digesting with Pirie's reagent (19), a mixture of 72% perchloric acid (1 vol.) and concentrated nitric (3 vol.) one-third saturated with copper nitrate. The digestions were carried out in 100 ml. Kjeldahl flasks on a sand bath and required 3–4 hours. Any remaining perchloric acid was boiled off over a free flame, leaving a dark colored dry residue. This was dissolved with the aid of 4 *M* HCl and the sulfur precipitated with BaCl<sub>2</sub> under standardized conditions. (The total sulfate in all samples was adjusted to 0.25 mM by the addition of the necessary amount of dilute H<sub>2</sub>SO<sub>4</sub>, due allowance being made for the sulfur in the tissue).

For determination of radioactivity the sulfate was filtered on to a good grade of filter paper. For this purpose a filter was constructed by cementing a porous alundum disk on the neck of a 2 liter filter flask. This disk supports the filter paper, and a glass cylinder held down on the paper with springs forms a kind of demountable Buchner funnel. This provides a filter onto which the BaSO<sub>4</sub> settles in an even layer of standard area. Since all conditions are exactly reproducible no difficulties are encountered due to self-absorption of the soft  $\beta$ -radiation of the sulfur by the precipitate. Standards can be digested, filtered and counted with an error not deviating from the mean by more than 5%. Most of this error is attributable to the counting technic (short time of counting).

The radioactivity determinations were made using a Geiger-Muller counter with a supported mica window bell type tube as described by Copp and Greenberg (20). Where necessary, corrections were applied for coincidence and for the decay of the radioactivity.

Labeled sulfate was determined in the combined medium and washes by precipitating BaSO<sub>4</sub> under the same conditions as described above. The labeled cystine remaining in the aliquot was diluted with 25 micromoles of cystine to avoid any significant contamination of the precipitated sulfate.

## RESULTS AND DISCUSSION

The results of incubating labeled cystine with liver slices and homogenates are shown in Table I. It is seen that the homogenates incorporated more cystine into the protein than did the slices, although the homogenates oxidized the cystine sulfur less readily to sulfate. One g. (wet weight) of homogenized liver incorporated nearly 6 micromoles of cystine into the protein. Analysis of the tissue showed about 70 micromoles of protein sulfur/g. of tissue. If we assume that the ratio between cystine sulfur and methionine sulfur is the same in rat liver as in beef liver (21) then of the 70 micromoles about 20 are from cystine sulfur. This means that about one-quarter of the cystine in the protein

is replaced in 2 hours or that free labeled cystine is either not washed out of the homogenate or additional cystine is combined with the protein and also that up to half of the optically inactive cystine was taken up by the proteins.

TABLE I

*Incubation of Cystine with Liver Sluces and Homogenates*

Medium: 6 ml. Krebs'-saline with 0.2% glucose.

Labeled Addendum: 10.5 micromoles cystine (21 micromoles sulfur) with 27,000 counts/min.

Gas Phase: 95% O<sub>2</sub>-5% CO<sub>2</sub>.

Incubation: 37-38°C.

Flask number <sup>a</sup>	Tissue wet weight	Time of incubation	Labeled sulfur found as	
			Protein	Sulfate
	<i>g.</i>	<i>min</i>	<i>micromoles</i>	<i>micromoles</i>
1	1.42	30	2.3	3.6
2	1.45	60	2.5	4.5
3	1.69	90	3.7	7.1
4	1.64	120	3.3	8.7
5	1.0	60	5.3	0.9
6	1.0	120	5.7	—

<sup>a</sup> Flasks 1-4 slices, flasks 5 and 6 homogenates.

The washing procedure was checked by adding 73 micromoles of labeled cystine to 1 g. of homogenate, then precipitating and washing in the usual way, with omission of any incubation. Under these conditions only 0.2% of the label was found in the protein. Therefore, the washing procedure is adequate.

There remains the possibility that the cystine is not bound with peptide bonds but with disulfide bonds. In order to test this, cystine was incubated with homogenates, then, the proteins were reduced with excess thioglycolic acid (1 g.) under the mild conditions employed by Stern and White (22). By this means it was found possible to remove two-thirds of the cystine from the protein as shown in Table II.

The amount of cystine reductively removed was not increased by adding cysteine ballast (1 millimole) to the thioglycolic acid and by adding thioglycolic acid to the first wash water.

Boiled homogenates likewise showed a lesser but still large cystine incorporation. Most of this cystine was likewise removed by reduction.

TABLE II

*Incubation of Cystine with Liver Homogenates*

Medium: 4 ml. Krebs'-saline with 0.2% glucose.

Labeled Addendum: 7.5 micromoles cystine with 19 360 counts min

Homogenate: 1.0 g.

Gas Phase: 95% O<sub>2</sub>-5% C<sup>18</sup>O<sub>2</sub>.

Incubation: 1.5 hours at 37-38°C.

Flask number	Conditions	$\mu M$ labeled sulfur found as	
		Protein	Sulfate
1	control	7.1	0.9
2	as 1	7.0	0.9
3	reduced with thioglycolic acid after incubation	2.7	1.0
4	as 3	2.7	1.4
5	heated to 100° before incubation	2.1	0.00
6	as 5	2.1	0.00
7	control	3.7	
8	reduced with thioglycolic acid and cysteine after incubation	1.3	
9	as 8	1.1	
10	heated to 100° for 15 min. before incubation	2.6	
11	as 10, plus reduction as in 8	0.7	

Boiling completely stopped sulfate formation under the conditions of these experiments.

## CONCLUSIONS

There is good reason to believe that protein synthesis will not proceed in homogenates under the conditions employed in these experiments, nor will it proceed after denaturing the homogenates by boiling. Uptake of cystine was observed under both of these sets of conditions. In addition, a large part of the cystine taken up could be removed by reduction. Consequently the major part of the cystine is not incorporated by peptide bond but by disulfide bond formation. So without modification the system is useless for the study of protein synthesis.

The cystine remaining in the protein may be peptide-bound but other possibilities remain. The reduction may not be complete or cysteine may be added to double bonds in the protein (23).

The smaller uptake of cystine by slices as compared with homogenates is probably due to slow penetration of cystine into the slices.

These experiments throw some doubt on the conclusions reached by Seligman and Fine (24) who, as a result of *in vivo* experiments, concluded that cystine is more rapidly incorporated into proteins than is methionine. Probably much of the cystine is bound by disulfide bonds and did not represent a rapid turnover of plasma proteins. Such experiments with cystine must be reinterpreted in the light of the work presented here.

Results of incubating methionine with slices and homogenates will appear in the following paper.

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#### SUMMARY

1. A synthesis of cystine is described. The method is suited to the preparation of cyst(e)ine with the sulfur labeled.
2. Incubation of labeled cystine with slices or homogenates results in an uptake of the label by the proteins.
3. The major part of the uptake is due to the formation of disulfide bonds, as shown by reduction studies.
4. The results make it necessary to proceed with caution in interpreting results of experiments with labeled cyst(e)ine.

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# Studies on Protein Synthesis *in vitro*. II. On the Uptake of Labeled Sulfur by the Proteins of Liver Slices Incubated with Labeled Methionine ( $S^{35}$ )

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## INTRODUCTION

In a previous communication (I) <sup>†</sup> we have shown that considerable difficulty is encountered in trying to use labeled cystine ( $S^{35}$ ) to study the synthesis of protein *in vitro*. Any incorporation of cystine into slice or homogenate proteins by peptide bond formation is masked by the relatively great uptake due to the formation of disulfide bonds.

The present communication deals with the use of methionine in experiments of a similar nature. There is a small uptake of labeled sulfur when rat liver slices are incubated with methionine but little or no uptake by homogenates. The uptake by slices varies with the concentration of labeled methionine used. A rapid conversion of methionine to cystine and to sulfate occurs. Part of the cystine so formed is also incorporated into the proteins but it is not known whether this incorporation is due to disulfide or peptide bond formation. It is very difficult to conceive of methionine being combined in the proteins by bonds other than peptide, so it has been assumed that the presence of labeled non-cystine sulfur (methionine) represents peptide bond formation and hence protein synthesis.

Various attempts to decrease the uptake of labeled cystine (and homocysteine) by adding cysteine or homocysteine were not successful.

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## EXPERIMENTAL

*Methods*

The incubations were carried out as previously described. Any modifications are indicated at the heads of the tables. Sulfate precipitations and radioactivity determinations were done as before.

*Cystine* was isolated from protein samples after 48 hour hydrolysis with 6 *N* HCl. The HCl was distilled off at reduced pressure and cystine isolated by the cuprous salt method of Rossouw and Wilken-Jorden (1). Free cystine was similarly isolated from aliquots of the wash waters from the slices. To minimize contamination with labeled methionine and sulfate 0.05 *mM* inactive methionine and 0.25 *mM* inactive sulfate were added to the aliquots before precipitation. The cuprous precipitates were well washed with acetate buffer (pH 4.5). Labeled cystine added to wash waters could be recovered by this method with slight loss, but labeled cystine added to slice proteins before hydrolysis could not be recovered after hydrolysis without loss (25% or more). Two to four *per cent* of this "lost" sulfur could be recovered as humin sulfur but the rest remained unaccounted for. In most experiments showing the distribution of the sulfur in the proteins this undetermined sulfur was included in the methionine fraction and the humin sulfur was ignored. Consequently, there is a consistent overestimation of the labeled methionine in the protein and an underestimation of the cystine. Homocystine sulfur would be in the cystine fraction but any significant contamination with this substance is improbable.

The *methionine* which remained unchanged was estimated in some experiments. To a convenient aliquot (10%) of the wash waters 200 mg. of methionine was added. The solution was reduced to a small volume and adjusted to about pH 6.5. Two volumes of ethyl alcohol were then added and the solution cooled in the refrigerator. The methionine which separated was recrystallized twice, washed with alcohol and weighed. This methionine was converted to sulfate and its radioactivity determined. Then if *W* mg. of isolated methionine show *C* counts per min. and *C*<sub>0</sub> is the total radioactivity present as methionine, the *per cent* methionine remaining is:

$$\% = \frac{C}{C_0} \times \frac{200}{W} \times 1,000.$$

In this calculation the weight of methionine in the aliquot is ignored. It is insignificant as compared with the 200 mg. methionine added as ballast.

The labeled methionine was synthesized according to Tarver and Schmidt (2).

## RESULTS AND DISCUSSION

The results of incubation of labeled methionine for 1.5–2 hours with liver slices from two month old rats are shown in Table I. In most experiments about 0.5 micromoles of methionine sulfur were taken up/g. of slices. There is in this weight of slices presumably about 50 micromoles of methionine (3). Therefore, about 1% of the methionine sulfur was replaced in 1.5–2 hours *if the label is all introduced as methionine*. Actually, all the label is not introduced as methionine (see Table

TABLE I

*Incubation of Methionine with Rat Liver Slices*

Medium: 5 ml. Krebs' saline with 0.2% glucose (rats 1-4, and 9-11).

6 ml. Krebs' saline with 0.2% glucose (rats 5-8).

Gas Phase: 95% O<sub>2</sub>-5% CO<sub>2</sub>.

Incubation: 37-38°C.

Rat number	Tissue wet weight	Incubation time	Substrate		Per cent labeled sulfur found as				Total recovered
					Protein	Cystine*	Sulfate*	Methionine*	
	g.	hrs	counts/min.	$\mu M$					per cent
1	1.33	1.5	50,000	38	1.1	11.6	3.4	—	—
1	1.97	1.5	50,000	38	1.3	12.4	4.2	—	—
2	0.68	1.5	22,000	21	1.3	—	3.8	—	—
2	0.71	1.5	22,000	21	1.2	13.5	4.1	84	103
2	0.83	1.5	22,000	210	0.51	14.6	0.8	80	96
3	0.55	1.5	22,000	21	0.58	16.2	4.4	85	106
3	0.58	1.5	22,000	84	0.45	17.4	0.9	69	88
3	0.69	1.5	22,000	210	0.37	10.8	0.6	90	102
4	0.75	1.5	22,000	21	1.4	17.2	5.0	—	—
4	0.96	1.5	22,000	21	1.7	21.4	5.8	—	—
4	0.90	1.5	22,000	84	1.1	20.0	2.2	72	95
5	1.32	2	41,000	55	0.7	5.6	2.8	—	—
6	1.87	2	41,000	55	1.1	6.8	2.6	—	—
7	1.57	2	32,000	121	0.6	5.2	0.9	—	—
8	1.02	2	32,000	121	0.4	5.0	1.2	—	—
9	1.03	2	39,000	33	2.1	—	8.8	—	—
9	1.05	2	39,000	33	2.1	8.5	5.5	—	—
10	0.69	2	39,000	33	0.8	10.9	4.9	—	—
11	0.81	2	39,000	33	1.2	7.6	6.1	—	—

\* In medium and washes from slices.

III). This replacement is very different from the uptake of cystine by homogenates incubated with cystine, and is of the order of magnitude which might be anticipated if the uptake is due to peptide bond formation.

Table I also shows that a very considerable fraction of the labeled sulfur is converted to cystine (5-20%) and 1-9% is completely oxidized to sulfate. The table also shows that much of the methionine remains unchanged. The recovery of the labeled sulfur varied from 88-106% of that added. This means that the constituents analyzed



account for the major portion of the labeled methionine; so that, under these conditions, no significant amounts of keto acid accumulate ( $\pm$ ).

In addition, Table I shows the results obtained when the concentration of methionine used is varied over a range of 4.2–42 micromoles/ml. of medium. An increase in the concentration of methionine causes an increase in the absolute amount taken up by protein and converted to sulfate but the *per cents* in the respective categories all fall. There is a decrease in the *per cent* taken up by protein by one-half or one-third when the methionine concentration is increased 10-fold. Cystine production is more nearly proportional to methionine concentration. The production of sulfate is almost independent of methionine concentration over the range investigated. From these experiments it is seen that it is an advantage to use methionine with high radioactivity when uptake by protein is being investigated.

TABLE II

*Effect of Homogenizing Tissue*

Medium: 5 ml. Krebs' saline with 0.2% glucose.

Gas Phase: 95% O<sub>2</sub>–5% CO<sub>2</sub>.

Incubation: 2 hrs. at 37–38°C.

Rat number	Tissue wet weight	Substrate methionine	Labeled sulfur found as:		
			Protein	Cystine	Sulfate
	g.	$\mu M$	$\mu M$	$\mu M$	$\mu M$ *
12	1.4 (slices)	12	0.5		2.8
12	1.1 (homogenate)	12	0.05		0.1
12	1.1 (homogenate)	12	0.10		0.2
13	2.4 (slices)	5	0.2	0.8	0.8
13	1.7 (homogenate)	5	0.005	0.08	0.12
13	1.5 (homogenate)	5	0.003	0.06	0.10
14	(slices)*	7	0.06	0.3	0.2
14	(homogenate) <sup>†</sup>	7	0.002	0.3	0.04

\* These preparations were incubated 1 hr. in 6 ml. total volume. Slices and homogenate were in comparable amounts.

Table II shows the effect of homogenizing the tissue. It is readily seen from these results that homogenizing reduces the uptake of methionine to a very low value. This is in agreement with the results of many other workers who have found that reactions requiring energy (endergonic reactions) do not go in homogenates without addition of

adenosine triphosphate or other similar substance. The observation shows that the uptake of methionine is concerned with the metabolic processes occurring in the cell. In this respect it is quite different from the uptake of cystine which is to some extent a non-enzymic reaction.

The last two homogenates (rat 14) were made with care to preserve the enzyme systems (5). Under these conditions cystine production persists although sulfate is not formed in significant amounts.

TABLE III  
*Distribution of Labeled Sulfur in Proteins*

Rat number	Tissue wet weight	Substrate methionine	Sulfur <sup>a</sup> found as protein	Distribution of labeled sulfur in proteins		Sulfur found as free	
				Cystine	Methionine	Cystine	Sulfate
	g.	$\mu M$	$\mu M$	per cent	per cent	$\mu M$	$\mu M$
13	2.4	13	0.5	41	59	2.0	2.5
15	0.88	21	0.3	30	70	1.6	0.9
16	1.25	20	0.1	26	74	1.0	1.4
17	1.96	20	0.4	31	69	2.0	1.7
9	0.99	33	0.5	19	81	3.1	3.1
10	0.95	33	0.4	17	83	3.1	4.1
11	0.93	33	0.2	19	81	3.0	2.1
5	1.32	55	0.3	25	75	3.1	1.5
6	1.87	55	0.6	32	68	3.0	1.5

\* These values are low due to the destruction of cystine by humin formation. If the labeled sulfur found as humin in 5 and 6 is included, the  $\mu M$  labeled sulfur found as protein becomes 0.5 and 0.7 respectively.

These results show that cystine is rapidly formed from methionine and previous results showed that cystine is rapidly taken up by proteins. Hence, it is important to determine whether the protein labeling is due to the uptake of cystine rather than of methionine. Consequently, liver slices were incubated with methionine and the proteins precipitated and washed. Cystine was isolated from the proteins as indicated under "Methods." In view of the fact that the labeled cystine content of the proteins is underestimated it is quite clear from Table III that a considerable part of the sulfur in the protein is introduced as cystine and *not* as methionine. In fact, in many cases it is probable that 50% or more of the labeling is due to cystine. In view of these results an attempt was made to depress the cystine uptake.

Two experiments were done. Relatively large amounts of either cysteine or homocysteine were added to the medium in addition to the labeled methionine. It was thought that by adding inactive cysteine any labeled cyst(e)ine produced would be so greatly diluted that its uptake would be negligible. However, the results in Table IV show that

TABLE IV

*Effect of Cysteine and Homocysteine*

Medium, gas phase, incubation as Table III.

Labeled Addendum: experiments with cysteine 32,000; with homocysteine 41,000 counts per min. of methionine.

Rat number	Tissue wet weight	Additions to medium	Labeled sulfur found as			Distribution of labeled sulfur in proteins	
			Protein <sup>*</sup>	Sulfate	Cystine	Methionine	Cystine <sup>*</sup>
	<i>g.</i>	$\mu M$	$\mu M$	$\mu M$	$\mu M$	<i>per cent</i>	<i>per cent</i>
20	1.57	120 methionine	0.5	2.2	6.1		
20	1.02	120 methionine 360 cysteine	0.3	0.5	9.5		
21	1.31	120 methionine	0.7	1.4	5.9		
21	1.35	120 methionine 360 cysteine	0.7	0.5	28.0		
5	1.32	55 methionine	0.5	1.5	3.1	55	45
5	1.35	55 methionine 110 homocysteine	0.08	0.4	2.3	45	55
6	1.87	55 methionine	0.7	1.5	3.0	59	41
6	1.73	55 methionine 110 homocysteine	0.1	0.4	2.3	51	49

\* These values include the labeled sulfur found as humin.

the uptake is apparently not decreased. The most remarkable effects of added cysteine are the increase in labeled cyst(e)ine formation and the decrease in labeled sulfate production. The first is probably due to sulfhydryl activation of the cysteine-producing system.

With homocysteine there is a decrease in the production of labeled cyst(e)ine but there is a much greater decrease in the protein labeling. This, taken together with the observed increase in the absolute amount of labeled cystine produced when labeled methionine concentration is increased (Table I), supports the assumption that homocysteine is an intermediate in the conversion of cystine to methionine. The reduction of protein labeling probably results from the methylation of the un-

labeled homocysteine to give unlabeled methionine causing a dilution of the labeling agent. The *per cent* of label in the protein found as cystine is actually not decreased.

Our thanks are due to Dr. Joseph Hamilton and the Radiation Laboratory of this University for generous supplies of radioactive sulfur.

### SUMMARY

1. Liver slices incubated with labeled methionine take up significant amounts of the label.
2. Homogenates do not take up the label under otherwise similar conditions.
3. The *per cent* of the label taken up depends on the concentration of the labeling agent. The *per cent* is greater when the concentration of methionine is low (20 micromoles/g. of slices in 5 ml. medium).
4. Part of the label is incorporated into the proteins as cyst(e)ine.
5. The production of cyst(e)ine is stimulated by added cysteine.
6. The addition of inactive homocysteine decreases the protein labeling.

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## The Presence of $\delta$ -Tocopherol in Hog Fats

We have previously described a method (1) for the determination of small amounts of mixed tocopherols by a modification of the Furter and Meyer procedure (2) and have given quantitative data on the tocopherol contents of various hog fats. In the modified procedure the nitric acid oxidation is followed by chromatographic separation of the red orthoquinones from other pigments, and the orthoquinones are then reacted with leuco methylene blue, making it possible to determine colorimetrically extremely low concentrations of the several previously known tocopherols. The method does not, however, distinguish between  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols. Recently, a new tocopherol termed  $\delta$ -tocopherol has been reported to be present in soybean oil to the extent of 30% of the total tocopherols (3). In the light of the properties reported for the new tocopherol, several unrecorded observations made by us in the studies of the tocopherol contents of hog fats are of interest.

One of the properties of the new tocopherol is that, after the initial rapid reaction in the Emmerie and Engel determination (4), it continues to reduce the iron-dipyridyl reagent slowly over an extended period of time. In our studies, it was found that the unsaponifiable fraction from rendered hog fats, after treatment by the modifications of Devlin and Mattill (5) and Hines and Mattill (6) and including the Parker and McFarlane treatment (7), also continued to reduce the Emmerie and Engel reagent slowly in much the same manner as reported for  $\delta$ -tocopherol.

It was also reported that the nitric acid oxidation of  $\delta$ -tocopherol yields a yellow-orange oxidation product. In chromatographing the nitric acid oxidation products of the tocopherol fractions of the rendered hog fats (1), there was obtained almost invariably an unexplained yellowish-orange fraction in the chromatographic tube which was closely associated with the red orthoquinones.

These and other somewhat less conclusive evidences make it clear that  $\delta$ -tocopherol may occur normally in the fats of hogs in small amounts, and, like the other tocopherols, is probably derived from the

diet. Further, although the yellowish-orange oxidation product appears not to oxidize leuco methylene blue readily, its separation chromatographically by a procedure similar to that we have used could possibly serve as the basis for a chemical method of determining  $\delta$ -tocopherol specifically and in small amounts in the presence of small amounts of the other tocopherols.

*The Hormel Institute,  
University of Minnesota,  
Austin, Minnesota.*

*October 29, 1946*

JACQUES R. CHIPAULT  
WALTER O. LUNDBERG

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### **d(-)Methylfolic Acid Displacing Agent for Folic Acid**

Sirs:

Displacing agents have been reported for every known member of the B complex with the exception of folic acid, recently identified, synthesized and designated by Angier *et al.* (1) as pteroylglutamic acid.

Methylfolic acid or N-(4-(((2-amino-4-hydroxy-7-methyl-6-pteridyl)-methyl)amino)benzoyl)-d(-)-glutamic acid was prepared by the method of Angier and coworkers (1) for folic acid by replacing 2,3-dibromopropionaldehyde by 2,3-dibromobutyraldehyde, and by replacing *p*-aminobenzoyl-l(+)-glutamic acid by *p*-aminobenzoyl-d(-)-glutamic acid. The methylfolic acid was a red-brown powder which appears to undergo slow decomposition without melting at temperatures much higher than 200 degrees.

Using *Strep. faecalis (lactis)* R (8043) in the folic acid assay technique (2), the results outlined in Table I were obtained.

d(-)Methylfolic acid is an effective displacer of folic acid. The ratio of inhibitor to metabolite for activity is approximately 150.

TABLE I  
*Methylfolic Acid as a Displacing Agent for Folic Acid*  
*Klett Readings*

Folic Acid Concentration $\gamma$ /10 ml.	Methylfolic acid concentrations $\gamma$ /10 ml.			
	0	1	10	100
0	70	53	55	66
0.0065	165	60	58	69
100.0065	188	171	175	164
1000.0065	222	223	220	235

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November 27, 1946

GUSTAV J. MARTIN  
 LEO TOLMAN  
 JACK MOSS

## Importance of Molecular Weight in Evaluation of Pharmacological Data

Sirs:


In the literature are to be found numerous papers in which the results of carefully executed experiments, designed to throw light on the relationship between chemical structure and pharmacological activity, are recorded; but conclusions which might then be legitimately drawn regarding such relationships are either not mentioned or are oftentimes given incorrectly. One possible cause of this situation may sometimes be a failure to realize the importance of molecular weight (1) in evaluating the results obtained, particularly with a series of chemically related compounds. For example, certain halogenated phenyl ethers have recently been tested (2) for antitubercular activity, and the *in vitro* data were expressed in milligrams (x) of compound "per cent" (pre-



sumably, that is, per 100 cc.) producing an observed *per cent* inhibition ( $y$ ) of growth of the bacilli, apparently in a constant period of time at constant temperature. It was then stated that "Replacement of nuclear iodine by other halogens did not lead to a definite pattern correlating chemical structure to antitubercular activity."

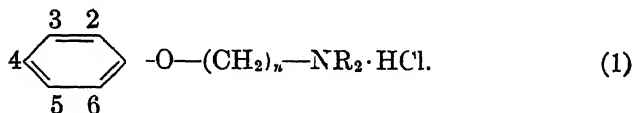
We have recalculated the respective concentrations as *micromoles* ( $x_1$ ) per 100 cc. and then arranged the compounds in ascending order of  $x_1$  needed to produce about the same *per cent* inhibition (as far as can be judged from the data available). For example, it will be seen that, for the first and third compounds in Table I, 3 mg.-% of either

TABLE I  
*Antitubercular Activity of Certain Halogenated Phenyl Ethers*

 $\text{O}-(\text{CH}_2)_n-\text{NR}_2 \cdot \text{HCl}$			Formula	M.W.	Effect on <i>in vitro</i> growth of tubercle bacillus		$\tau_1$ ( $r$ in terms of $\mu M$ )	$\log y_{x_1}$
-phenoxy	$n$	R			Dose, $r$ mg.-%	Inhibition, $y$ %		
2,4,6-triiodo-	2	methyl	$\text{C}_{10}\text{H}_{12}\text{I}_3\text{NO} \cdot \text{HCl}$	579.43	3	90	5.2	0.377
2,4,6-tribromo-	2	methyl	$\text{C}_{10}\text{H}_{12}\text{Br}_3\text{NO} \cdot \text{HCl}$	438.42	3	76.6	6.8	0.275
2,4,5-trichloro-	2	methyl	$\text{C}_{10}\text{H}_{12}\text{Cl}_3\text{NO} \cdot \text{HCl}$	305.04	3	92.1	9.8	0.106
2,4,6-trichloro-	2	methyl (no HCl)	$\text{C}_{10}\text{H}_{12}\text{Cl}_3\text{NO}$	288.58	5	95.5	18.6	0.106
2,4,6-triiodo-	3	ethyl	$\text{C}_{12}\text{H}_{14}\text{I}_3\text{NO} \cdot \text{HCl}$	621.50	3	96	4.5	0.411
2,6-diiodo-4-bromo-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{BrI}_2\text{NO} \cdot \text{HCl}$	560.48	3	95	5.4	0.369
2,6-diiodo-4-chloro-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{ClI}_2\text{NO} \cdot \text{HCl}$	516.02	3	86	5.8	0.333
2,4-diiodo-6-methyl-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{I}_2\text{NO} \cdot \text{HCl}$	495.59	3	88	6.1	0.321
2,6-diiodo-4-methyl-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{I}_2\text{NO} \cdot \text{HCl}$	495.59	3	62	6.1	0.296
2,4,6-tribromo-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{Br}_3\text{NO} \cdot \text{HCl}$	466.47	3	56.2	6.4	0.272
2,4,6-triiodo-3-methyl-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{I}_3\text{NO} \cdot \text{HCl}$	618.48	5	95.4	8.1	0.245
					5	97.7		0.246
2,4,5-trichloro-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{Cl}_3\text{NO} \cdot \text{HCl}$	333.09	2	61.5	6.0	0.293
					5	67.2	15.0	0.122
					5	72.1		0.124
2,4,6-trichloro-	2	ethyl	$\text{C}_{12}\text{H}_{14}\text{Cl}_3\text{NO} \cdot \text{HCl}$	333.09	5	52.2	15.0	0.114
2,4,5-trichloro-	1	ethyl	$\text{C}_{11}\text{H}_{13}\text{Cl}_3\text{NO} \cdot \text{HCl}$	319.00	5	82.6		0.123
					5	0.0	15.7	
2,4,6-triiodo-3-methyl-	2	<i>n</i> -butyl	$\text{C}_{17}\text{H}_{20}\text{I}_3\text{NO} \cdot \text{HCl}$	677.61	3	20	4.4	0.294
2,4,3-trichloro-	2	<i>n</i> -butyl	$\text{C}_{16}\text{H}_{18}\text{Cl}_3\text{NO} \cdot \text{HCl}$	388.19	3	97	7.7	0.238

produces about the same *per cent* inhibition, but the molarity for the third compound is almost double that for the first; hence, presumably, the first compound is actually the more active. We have also calculated for each compound the increment in  $\log y/\mu M$ . We then find what is apparently a beautiful correlation between chemical structure and antibacterial activity for all the substances of this type for which *in vitro* figures were given by Burger *et al.* (2) (see Table I).

The compounds studied may be regarded as having three components, namely: a (substituted) phenoxy group joined through an aliphatic chain to a tertiary amino group, as in 1.



The most active drug in the table has a three-carbon chain; the only recorded compound with a single carbon in the chain is devoid of activity; all the others have a two-carbon chain. This suggests that an "insulating" chain of at least three carbon atoms is required for greatest activity, a reasonable conclusion in view of the calculations of Branch and Calvin (3).

Considering the compounds in which  $n$  equals 2, it may be seen from Table I that, if R is a methyl group, the antitubercular activity decreases as the electronegativity of the halogen substituents is progressively changed; thus, the order is 2,4,6-triiodo-, 2,4,6-tribromo-, and 2,4,5-trichloro-. Similarly, when R is an ethyl group, the activity again decreases in the order, 2,6-diiodo-4-bromo, 2,6-diiodo-4-chloro-, 2,4,6-tribromo-, and 2,4,6-trichloro-. The activities of the 2 di-( $n$ -butyl)-amino derivatives recorded are in the order: 2,4,6-triiodo-3-methyl- and 2,4,5-trichloro-.

"The decreasing order of negativity of the halogens is F, Cl, Br, I" (4), but as regards the effect on the electron density at position 1 this order appears to be reversed in aromatic substitution. Since, in this series, it would seem that the greater the electronegativity (in the sense implied by Hammett (5)) of the substituents on the benzene ring, the greater the activity of the compound, substitution (on this ring) of groups successively even more electronegative (5) may be expected to result in compounds having activities successively greater than those of the iodine-substituted analogs.

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## Book Reviews

**A Textbook of Biochemistry.** By PHILIP H. MITCHELL. McGraw-Hill Book Company, Inc., New York, 1946 (first edition). 640 pages. Price \$5.00.

The subject of biochemistry has become so vast that it is impossible to give more than a general survey in a standard sized text. Mitchell's "A Textbook of Biochemistry" suffers from this broadness of scope and the attendant space limitations. It is perforce an abbreviated and somewhat uncritical presentation, with too little consideration of experimental evidence and controversial topics. Aside from this general criticism, which, by the way, can be leveled at other similar works, the book is admirably organized and clearly presented. It is designed as a general biochemistry text with the emphasis slanted toward subjects of special importance to beginning medical students. Clinical and pathological chemistry is, however, not emphasized.

The organization of the book follows closely more or less classical lines, with perhaps somewhat more than usual emphasis on nutrition. The chapters on the chemistry of carbohydrates, fats, proteins, and nucleoproteins are clearly presented in a lucid though concentrated form. The chapter on vitamins, in the opinion of the reviewer, leaves something to be desired. It contains minor inaccuracies, and the general topic of vitamin antagonism by structurally related compounds has not been considered. One also wonders why unconventional spelling for some of the B complex vitamins was adopted. The chapters on digestion, absorption, and detoxication are nicely done, but necessarily brief. The role of phosphorylation in the absorption of carbohydrates is not considered here, and a few other topics properly associated with these sections are omitted.

The chapter on blood emphasizes primarily blood proteins, blood clotting and hemoglobin, and slights important considerations on the significance of some other blood constituents. It is followed by an excellent chapter on the chemistry of respiration and acid-base balance. The chapters on biological oxidation, energy metabolism and intermediary metabolism are brief, but well done. The remaining chapters on urine, dietetics, tissue chemistry, hormones, and chemotherapy are well-balanced and lucid.

It is possibly clear from the foregoing comments that the principal criticism of this book lies in the extent to which it has been necessary to condense the multitude of facts and principles encompassed by modern biochemistry. Compared to other available general biochemistry texts, however, Mitchell's "A Textbook of Biochemistry" stands in a favorable light.

RICHARD J. WINZLER, Los Angeles, Calif.

**A Textbook of Biochemistry.** By BENJAMIN HARROW, Professor of Chemistry in the City College, College of the City of New York. Fourth Edition, W. B. Saunders Company, Philadelphia and London. 1946. 592 pp. with 144 illustrations. Price \$4.25.

The publication of the fourth edition of this well known and widely used textbook, the first edition of which appeared in 1938, is evidence of the approval which the

author's presentation has received from teachers of biochemistry throughout the country. The new book follows, in general, the lines which have been so successful in earlier editions. The inclusion of material of more clinical value will assist materially in helping the student of medicine in correlating the work in biochemistry with that of the other preclinical medical sciences and will increase its value as a reference book for the clinical years of the medical curriculum.

An innovation is the inclusion in the Appendix of certain subjects which, in the opinion of the author, should be included in an elementary text but which are "possibly too advanced for the general student." Included here are such topics as the evidence for the structure of cholesterol, the synthesis of glutathione, thiamine, androsterone and details of the methods of isolation of certain enzymes and hormones. Included also is the series of reactions which are concerned in the manufacture of nylon, a presentation whose inclusion in a text of biochemistry seems difficult to justify. It is believed that in the interests of the very considerable group of students who probably constitute the chief users of this book, students of medicine and the biological sciences, other syntheses could be similarly relegated to an appendix or omitted altogether, e.g. those of serine (p. 51), methionine and related sulfur compounds (p. 54) and phenylalanine (p. 56).

The chapter on the chemistry of carbohydrates while, in general satisfactory, might be of more value for the chief group for whom this book is designed. Is "gamma" glucose a term of sufficient importance today to warrant its use in a section heading, when as pointed out in the discussion of this section, the terminology of "furanose" sugars has been generally accepted? In view of the increasing biological importance of uronic acids, the limited discussion of this group and its derivatives, the "polyuronides" could well be expanded.

The discussion of vitamins is of special excellence. The inclusion in the chapter on nucleoproteins of up-to-date material on the chemistry of the cell, the chromosome, the gene and of virus proteins offers a new and challenging outlook on a subject which, too often in the past, has been one of pure organic chemistry with little application to life in the eyes of the student of biological sciences.

The volume should be of greater value than the preceding editions and will undoubtedly continue to be used extensively. Typographic errors are relatively few. Particularly confusing to the inexperienced student is the use of "erythrodextrin" in lines 11 and 12 of page 24 in place of the correct "amylopectin."

HOWARD B. LEWIS, Ann Arbor, Michigan

**Currents in Biochemical Research.** Edited by DAVID E. GREEN. Interscience Publishers, Inc. New York, N. Y. 1946. viii + 486 pp. Price \$5.00.

In 1936 Joseph Needham and David E. Green, acting on behalf of an organizing committee, invited a number of biochemists who had been associated with Sir F. G. Hopkins, to celebrate his 75th birthday by presenting to him a book of essays on modern biochemistry. It was suggested to the contributors that the essays should deal with modern tendencies in research; that they should not be reviews of the literature or descriptions of experimental work, but be of a suggestive and provocative nature, thus providing an opportunity for the inclusion of items of a more general and perhaps speculative sort than can be included in the ordinary contributions to

the scientific journals. The result of this enterprise was the now well-known volume "Perspectives in Biochemistry." It had an enthusiastic reception and had to be reprinted to meet the demand. No doubt it was the success of this book which encouraged Dr. Green, after an interval of ten years, to collect a similar volume of essays on modern biochemistry. According to the introductory remarks of the editor "Currents in Biochemical Research" represents an attempt to describe, in as simple language as possible, the important recent developments in various fields of biochemistry and to speculate on the most likely paths of future progress. "The aim of these essays has been to excite the imagination and to provide glimpses of some of the fascinating horizons of biochemical research. However, no popularizations were intended. The various contributors were asked to write simply and provocatively but without sacrifice of scholarship."

The book contains 31 articles by 34 authors. The authors and titles are as follows: G. W. Beadle, "The Gene and Biochemistry"; W. M. Stanley, "Viruses"; H. Gaffron, "Photosynthesis and the Production of Organic Matter on Earth"; René J. Dubos, "The Bacterial Cell"; D. R. Hoagland, "The Nutrition and Biochemistry of Plants"; C. A. Elvehjem, "Biological Significance of Vitamins"; Karl Folkers, "Some Aspects of Vitamin Research"; Donald D. Van Slyke, "Quantitative Analysis in Biochemistry"; Joseph S. Fruton, "Enzymic Hydrolysis and Synthesis of Peptide Bonds"; Fritz Lipmann, "Metabolic Process Patterns"; David E. Green, "Biochemistry from the Standpoint of Enzymes"; Severo Ochoa, "Enzymic Mechanisms of Carbon Dioxide Assimilation"; B. A. Houssay, "Hormones"; Leonor Michaelis, "Fundamentals of Oxidation and Reduction"; Herman M. Kalckar, "Mesomeric Concepts in the Biological Sciences"; Max A. Lauffer, "Viscometry in Biochemical Investigations"; D. Rittenberg and David Shemin, "Isotope Technique in the Study of Intermediary Metabolism"; Karl Meyer, "Mucolytic Enzymes"; Konrad Bloch, "Some Aspects of Intermediary Metabolism"; Gregory Pincus, "The Steroid Hormones"; Kenneth V. Thimann, "Plant Hormones and the Analysis of Growth"; David Nachmansohn, "Chemical Mechanism of Nervous Action"; D. W. Woolley, "Some Aspects of Biochemical Antagonism"; Rollin D. Hotchkiss, "Chemotherapy: Applied Cytochemistry"; Arnold D. Welch and Ernest Bueding, "Biochemical Aspects of Pharmacology"; Charles L. Hoagland, "Some Biochemical Problems Posed by a Disease of Muscle"; Surgeon Captain C. H. Best, "Physiology and Biochemistry"; I. Fankuchen and H. Mark, "X-Ray Diffraction and the Study of Fibrous Proteins"; Michael Heidelberger, "Immunochemistry"; W. H. Sebrell, "Social Aspects of Nutrition"; L. C. Dunn, "Organization and Support of Science in the United States."

The many distinguished names in this list speak for themselves. They vouch for the highest standard and make it unnecessary to praise the book. The various authors have tackled the task in different ways. Some have preferred to give straightforward summaries of their particular fields, while others have followed in varying degrees the invitation of the editor to speculate. All the articles are well worth reading; many of them are fascinating. It would hardly be fair to select some for special commendation. The book will appeal alike to senior students, to teachers, and to the experienced research worker.

There is one small criticism which the reviewer feels ought to be made. The book contains an error which occurs in many other biochemical books (including Annual

Reviews of Biochemistry and many text books). It concerns the structural formula of adenylic acid and related compounds. On p. 179 a formula of adenosine triphosphate is given where the pentose is *l*-lyxose instead of *d*-ribose, although in the same article on p. 169 the correct structure is given for the pentose in the formula of the diphosphopyridine nucleotide. There are misprints in the formulae II, p. 230 and V, p. 231.

H. A. KREBS, Sheffield, England.

**The Collected Papers of C. S. Hudson, Volume I.** Edited by R. M. HANN and N. K. RICHTMYER, National Institute of Health, U. S. Public Health Service, Bethesda, Maryland. Academic Press Inc., Publishers, New York, N. Y. Price \$15.00.

The honor of publishing their collected papers is bestowed on the recognized leaders of experimental science only. If such publication is arranged during the lifetime of the author this clearly expresses the conviction that his writings are indispensable and that their value appears everlasting.

On the occasion of the 65th birthday of Claude Silbert Hudson and the Hudson Celebration of the Division of Sugar Chemistry and Technology of the American Chemical Society at Chicago in September 1946 the first volume of Hudson's collected papers just appeared. Its 898 pages contain 118 papers by Hudson and his associates, a total of 247 having been published up to June 1945.

Raymond M. Hann and Nelson K. Richtmyer, both of them close to Hudson and from the National Institute of Health, Bethesda, Maryland, took care of the intelligent and lucid arrangement of the mighty subject matter. W. W. Pigman and W. L. Evans for the Publication and Finance Committee of the American Chemical Society saw to it that the necessary means for the publication of the compilation were available. Academic Press, Inc., Publishers, New York, N. Y., are responsible for the excellent presentation and the perfect letterpress. A portrait of Hudson embellishes this first volume, and a most attractive autobiography enhances the value of the book by acquainting the younger generation and posterity with the evolution of a great scientist and the influence exerted on his development by his environment.

CARL NEUBERG, New York, N. Y.

## ERRATA

Volume 11, Number 2, page 372, line 10:

"Acid phosphatase" should read "Alkaline phosphatase"

Volume 11, Number 3, page 439, third column heading:

"micrograms" should read " $\pm$ micrograms"

# Carbohydrate Metabolism in Tissue Homogenates

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Received November 7, 1946

## INTRODUCTION

Previous work on the anaerobic glycolysis of nervous tissue (1, 2) has indicated how tissue preparations may be reinforced in such a way as to make possible the measurement of their maximal potential activity.

We consider here the requirements for maximal aerobic activity with glucose as substrate, and extend the investigation to homogenates of liver, which differs from brain in many respects. Brain has been investigated in greater detail, particularly because of its extremely high rate of aerobic glycolysis, which was an interesting and unexpected result of our findings.

## MATERIALS AND METHODS

The animals used were albino rats from our colony, of the Sprague Dawley and Sprague Dawley  $\times$  La Jolla strains, and occasional hooded rats from the colony of the Department of Agricultural Biochemistry. The animals were lightly anesthetized with ether, decapitated, and the brain and liver quickly removed and weighed. "Brain," as here employed, refers to cerebrum and cerebellum taken together.

Ice cold distilled water was added in the proportion of 4 ml. to each gram of tissue, and the tissues were then homogenized in a glass homogenizer of the Potter-Elvehjem type (3). In the case of liver, it was found best to cut the tissue into small pieces with a sharp scissors before grinding, since otherwise a smooth homogenate is difficult to obtain.

Manometric studies were conducted with the usual Warburg apparatus at a temperature of 38.2°C., in 20-ml. conical reaction vessels equipped with side-arms. In general, all materials except the tissue homogenate were placed in the main compartment, the tissue being placed in a side-arm. After gassing and equilibration, the tissue

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was washed out of the side-arm with the contents of the main compartment, and readings were begun 3-5 minutes thereafter.

Oxygen consumption and  $\text{CO}_2$  production in air were determined by the direct method of Warburg (4). Aerobic and anaerobic glycolysis were determined as usual (4) in bicarbonate buffer in equilibrium with an atmosphere containing 5%  $\text{CO}_2$ . All determinations were run in duplicate.

The total fluid volume in each cup was made up to 1 ml. with distilled water, although occasionally it was necessary to exceed this volume slightly.

Cytochrome c was obtained from horse heart by Potter's modification of the method of Keilin and Hartree (5). It was used in a  $3 \times 10^{-4}$  M solution, being standardized manometrically and spectrophotometrically against a known sample which was very kindly sent to us by Dr. Van R. Potter of the University of Wisconsin.

Adenosine triphosphate was obtained from rabbit skeletal muscle (6) as the Ba salt, the purity by ribose and phosphate determinations being in excess of 95%. Hexose diphosphate was obtained as the commercial Ba salt, of purity in excess of 85%. Both of these compounds were converted into the K salts, with the concomitant removal of Ba, when the stock solutions were made up.

Coenzyme I was prepared by the method of Williamson and Green (7), the purity in different preparations varying between 50% and 65%.

All solutions were neutralized before use.

## RESULTS

### *Conditions for Maximal Activity in Tissue Homogenates*

*a. Brain.* The requirements for maximal anaerobic glycolytic activity for brain had already been worked out (1, 2), and essentially the same method was used here.

If respiration of brain tissue began in the same way as glycolysis and passed through the Meyerhof cycle on the way to pyruvate, which there seemed no reason to doubt, respiratory activity would require all the co-factors needed for glycolysis, such as adenosine triphosphate (ATP), hexose diphosphate (HDP), Coenzyme I (DPN),  $\text{Mg}^{++}$ , and nicotinamide. In addition, it seemed likely that the dilution of the tissue involved in homogenizing would introduce a requirement for Cytochrome c. It has also been supposed (8) that the oxidative system requires catalytic amounts of fumarate or succinate.

The results of some experimental series which indicate the value of the various additions are given in Table I. The numerical values are expressed as  $\text{Q}_{\text{O}_2}$  (microliters of  $\text{O}_2$  consumed/hr./mg. dry tissue). Each value is the mean of duplicates.

Data such as these permit no doubt concerning the necessity for the major additions. In particular, the omission of DPN gives strikingly

TABLE I  
*Requirements of Respiratory System of Brain*

	QO <sub>2</sub>
Experiment 1: Complete System	18.5
No HDP	8.0
No ATP	9.8
No DPN	3.3
No Cytochrome c	8.3
No Mg	18.0
No nicotinamide	13.5
No fumarate	15.5
No HDP or glucose	6.5
Experiment 2: Homogenate (complete system)	17.3
Extract (complete system)	7.0
Homogenate + Mn 0.008 M (No Mg)	8.3

Medium: Glucose 0.028 M, HDP 0.005 M, ATP 0.0007 M, DPN 0.001 M, Cytochrome c 0.00006 M, Fumarate (K salt) 0.0016 M, nicotinamide 0.04 M, MgCl<sub>2</sub> 0.008 M, K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.4) 0.04 M. 0.1 ml. of homogenate or extract. Fluid volume 1.0 ml. Omissions from medium as indicated in table.

low values. This is in agreement with the known rapid rate of destruction of DPN in surviving tissues (9, 10, 11) and its well-known necessity for carbohydrate metabolism. The only addition which seems dubious is Mg<sup>++</sup>; it is possible that the aerobic requirement is smaller than the anaerobic, and is satisfied by the concentration initially present in the homogenate.

In Experiment 2 the rates of homogenates are compared with those of extracts, which are prepared by centrifuging the homogenates. The low value for extract, in marked contrast to the high rates obtained for glycolysis with extracts (2), is probably due to deficiency in cytochrome oxidase, which is associated with the large particles. It was also found that the substitution of Mn<sup>++</sup> for Mg<sup>++</sup> markedly depresses the rate.

In view of the common supposition that the validity of an enzyme assay requires a linear relationship between activity and enzyme concentration, experiments were performed in which different quantities of homogenate were used, the other additions being the same, and the total volume 1 ml. A typical result is given in Table II, which indicates that the system comes close to satisfying the requirement of linearity.

*b. Liver.* Liver presented somewhat more difficulties than brain, and differed from it in a number of respects. The major peculiarity was the

TABLE II  
*Respiration as Function of Brain Concentration*

	$Q_{O_2}$
Homogenate 0.05 ml.	16.5
0.1 ml.	16.8
0.15 ml.	15.8
0.2 ml.	14.8

fact that  $Mn^{++}$ , rather than  $Mg^{++}$ , appeared to be the ion required for  $O_2$  uptake (Table III), while the reverse was true for anaerobic gly-

TABLE III  
*Requirements of Respiratory System of Liver*

	$Q_{O_2}$
Complete System	16.0
No Cytochrome c	7.3
No HDP	3.5
No ATP	14.9
No DPN	1.8
No fumarate	3.5
No Mn	12.2
No glucose or HDP	4.2

Basal medium as in Table I, except for 0.008 *M*  $MnCl_2$  instead of  $MgCl_2$ .

TABLE IV  
*Requirements of Anaerobic Glycolytic System of Liver*

	$Q_L^{N_2}$
Experiment 1: Complete System	24.1
No HDP	5.5
No ATP	23.1
No DPN	8.2
Experiment 2: No Mg or Mn	19.9
Mg 0.008 <i>M</i>	23.9
Mn 0.008 <i>M</i>	13.3
Mg + Mn, each 0.008 <i>M</i>	16.6

Basal medium as in Table I; Cytochrome c and fumarate omitted; bicarbonate buffer.

colysis (Table IV). Glycolysis is expressed as  $Q_L$  (microliters of lactic/hr./mg. dry tissue);  $Q_L^{O_2}$  and  $Q_L^{N_2}$  refer to aerobic and anaerobic conditions respectively.

Anaerobic glycolysis of liver is less than half that of brain (brain  $Q_L^{N_2}$  about 60; see also comparison in Table VII). The anaerobic ac-

tivity of liver shows a much more marked decline with time over a 60-minute period than that of brain or the respiration of liver. Various experiments were performed in the attempt to get higher and steadier activity. A higher concentration of nicotinamide (0.08 *M*) appeared to be more nearly optimal for liver. Further increase was obtained by doubling the concentrations of  $Mg^{++}$  and HDP. Increase of ATP concentration was without effect.

The variation of respiratory activity with concentration of tissue in the complete system is shown in Table V, which also shows the effect of Mn. The Mn column shows a fair proportionality with amount of tissue, while with Mg the rate declines with increasing amount of tissue.

Table VI presents similar data for anaerobic glycolysis.

TABLE V  
*Respiration as Function of Liver Concentration*

		$Q_{O_2}$	
		0.008 <i>M</i>	0.008 <i>M</i>
		Mg	Mn
Homogenate	0.05 ml.	12.0	11.6
	0.1 ml.	13.1	11.5
	0.15 ml.	10.3	12.5
	0.2 ml.	9.3	11.6
Medium as in Table III.			

TABLE VI  
*Anaerobic Glycolysis as Function of Liver Concentration*  
(A: Nicotinamide 0.04 *M*, Mg 0.008 *M*, HDP 0.005 *M*;  
B: Nicotinamide 0.08 *M*, Mg 0.016 *M*, HDP 0.01 *M*.)

		$Q_E^{21}$	
		A	B
Homogenate	0.05 ml.	26.6	29.1
	0.1 ml.	16.8	28.4
	0.15 ml.	10.4	20.4
	0.2 ml.	8.4	17.8

Basal medium as in Table IV, except as noted above.

It has not thus far been possible to obtain satisfactorily linear variation with enzyme concentration in liver glycolysis. The steadiness of the rate of glycolysis over the full 60-minute period has proved quite variable from one animal to another. Doubling the amount of DPN

was tried without significant effect. It is impossible to say whether this situation is inherent in the nature of the system or whether it is due to some factor which has not yet been taken into consideration.

*c. Mouse Brain Homogenates.* A few experiments were performed with mouse brain, using mice of the Bittner ZBC strain. It was found that homogenates prepared in the same way as those of rat brain were equally active. The necessity of Cytochrome c in particular was demonstrated. Typical values, the averages of duplicate determinations, for 60 minutes are:

With Cytochrome c	67 microlitres of O <sub>2</sub>
Without Cytochrome c	45

A paper by Racker and Krinsky (12) on mouse brain homogenates appeared recently, which included some data on oxygen uptake in substantial agreement with our own as to order of magnitude. However, Racker's preparations were not reinforced with Cytochrome c. We are unable to account for this discrepancy except by supposing that a strain difference is involved, since Racker used Swiss albino mice. Similar strain differences in rates of anaerobic glycolysis between Swiss albinos and mice of other strains have, in fact, been observed by M. F. Utter (private communication).

### *Respiration and Glycolysis of Tissue Homogenates*

In Table VII are recorded the results of 3 typical experiments, in each of which respiration, aerobic glycolysis, and anaerobic glycolysis were determined simultaneously on homogenates of brain and liver from one animal. Aerobic glycolysis was calculated, as usual, from the

TABLE VII  
*Respiration and Glycolysis of Homogenates*

	Q <sub>O<sub>2</sub></sub>	Q <sub>CO<sub>2</sub></sub>	R. Q.	Q <sub>L</sub> <sup>N<sub>2</sub></sup>	Q <sub>L</sub> <sup>O<sub>2</sub></sup>	M.O.Q.*
<i>Brain:</i>						
Experiment 1	15.0	13.3	0.88	63.3	65.1	(0)
Experiment 2	15.3	11.0	0.72	61.3	59.1	0.44
Experiment 3	19.0	14.8	0.78	74.0	72.0	0.32
<i>Liver:</i>						
Experiment 1	11.8	13.5	1.13	15.5	4.2	2.9
Experiment 2	10.2	10.8	1.07	22.0	5.5	4.9
Experiment 3	9.1	10.2	1.12	20.6	3.6	5.6

\* (Meyerhof Oxidation Quotient =  $3(Q_L^{N_2} - Q_L^{O_2})/Q_{O_2}$ )

Media as indicated in previous tables.

gas output in bicarbonate buffer and the respiratory data, on the assumption that  $O_2$  consumption and respiratory  $CO_2$  production are the same as in phosphate buffer.

The least certain of these figures are the respiratory  $CO_2$  and the R. Q., because of the uncertainties in making a correction for retention. Nevertheless, the precise value of the R. Q., provided it is somewhere in the neighborhood of 1.0, has little effect on the calculated value of the aerobic glycolysis, particularly in the case of brain.

The high aerobic glycolysis of brain, which has been reproduced in some scores of experiments, is one of the most striking findings in these studies. It is in sharp contrast with liver, a contrast which is emphasized by the calculated values of the Meyerhof oxidation quotient in the last column of Table VII. Not only do the data show that about 12 molecules of glucose are glycolyzed for every one which is oxidized (*e.g.*,  $(65.1/2) \div (15.0/6) = 13$ , Table VII, first row); but comparison with the anaerobic activity indicates the almost complete absence of any Pasteur effect.

The finding of a high aerobic glycolysis and absence of Pasteur effect in brain tissue is at variance with virtually all previous reports (13, 14, 15), except for one study by Haarmann and Brink (16). Typical of previous findings are the figures of Dixon (cited by Quastel (13)), who finds a  $Q_{O_2}$  of 8.3 and a  $Q_{CO_2}^a$  of 2.8, compared with the corresponding values for the averages of the 3 experiments of Table VII of about 16.4 and 65.4 respectively.

In view of the apparent contradiction, it seemed important to check the manometric findings by direct determinations of lactic acid. The results appear in Table VIII. Lactic acid was determined in a  $CdSO_4$ -NaOH filtrate by the method of Barker and Summerson (17), while

TABLE VIII  
*Aerobic Glycolysis, Esterification of Phosphate, and Consumption of  
Glucose by Brain ( $\mu M$ )*

Buffer	Glucose Consumed	Lactate Produced	Inorganic Phosphate Disappeared	Glycolytic $CO_2$ Produced	$\frac{1}{2} O_2$ Consumed
Bicarbonate	8.3	9.7	2.2	10.5	—
Phosphate	—	10.5	1.8	—	0.6

Contents of Warburg reaction vessels at 0 and 60 minutes deproteinized and analyzed as described in text.

glucose was determined in a tungstic acid filtrate by the method of Folin and Malmros (18). In one or two cases, inorganic phosphate was also determined in a trichloroacetic acid filtrate by the method of Lohmann and Jendr ssik (19).

To make certain that the results were not due to some specific effect of the bicarbonate buffer, determinations of lactic acid were also made on the contents of reaction vessels which had been used for the measurement of  $O_2$  uptake and respiratory  $CO_2$ , and in which the usual 0.04  $M$  phosphate buffer was used.

The agreement in Table VIII is satisfactory in several respects. Direct lactate determination and glycolytic  $CO_2$  check well (9.7 and 10.5  $\mu M$ ); lactate produced in phosphate and in bicarbonate buffer is substantially the same (10.5 and 9.7  $\mu M$ ), as is phosphate esterification (1.8 and 2.2  $\mu M$ ). Moreover, the glucose equivalents of lactate and respiration (4.8 and 0.6) together with the phosphate esterified (2.2) give 7.6  $\mu M$ , or 91.5% of the glucose consumed. If half the esterified P is used in the calculation, the total is 6.5, a recovery of 78.3%.

The strikingly high glycolytic capacity of the brain, both aerobically and anaerobically, raises the question why the  $O_2$  consumption should be no higher than it is, assuming that the preliminary stages of oxidation pass through essentially the usual Meyerhof scheme. Accordingly, some experiments were performed by way of a preliminary orientation in determining the rate-limiting step of the respiration. Table IX presents the first of these, dealing with respiration in the presence of glucose and pyruvate. Data on liver are included by way of comparison. Pyruvate was used in a strength of 0.05  $M$ , and NaF at 0.01  $M$ .

TABLE IX  
*Effect of Fluoride and Pyruvate on Respiration*

	Glucose + 0.05 $M$ Pyruvate	0.05 $M$ Pyruvate	Glucose	Glucose + 0.01 $M$ NaF
<i>Brain:</i>				
$QO_2$	18.5	25.8	20.4	23.0
$QCO_2$	16.0	26.3	16.0	8.5
<i>Liver:</i>				
$QO_2$	6.6	9.8	12.0	14.7
$QCO_2$	13.3	18.0	14.2	15.7

Media as indicated in previous tables, except for additions of pyruvate and fluoride.

The fact that brain is no more active on glucose and pyruvate together than on glucose alone, while on pyruvate alone it is a little but not much more active than on glucose, suggests that pyruvate oxidation might be the rate-limiting step, or at least one such step. The fact that NaF strongly depresses  $\text{CO}_2$  production, while its effect on oxidation is stimulating if anything, does not contradict this; it would be a reasonable result if triose phosphate oxidation were sufficiently active, and were the principal pathway of  $\text{O}_2$  consumption.

It is worth noting in this connection that observations on pyruvate oxidation in the absence of HDP not only yielded low values for the 60-minute period, but in most cases activity died out after the first 15 or 20 minutes. Since the  $5 \mu\text{M}$  of HDP usually added, if oxidized only to the phosphoglycerate stage, would permit the consumption of 112 microlitres of oxygen, it seems possible that oxidation of pyruvate by our brain preparations is negligible.

#### DISCUSSION

These experiments were designed to arrive at tissue preparations of high activity, in which factors of structure, permeability, and concentration of substrates and co-factors would, as far as possible, not be limiting. The data attest that this purpose has in considerable measure been achieved.

The brain preparations, for instance, exhibit  $Q_{\text{O}_2}$  values lying between 15 and 20. Except for the recent data of Racker (12), which deal with mouse and not with rat brain, the only values in the literature which approach this range are some figures calculated from *in vivo* measurements (13), which are fraught with considerable uncertainty due to the difficulties of measuring the blood flow through the brain. The highest values found for *in vitro* experiments are 19.1 (20) and 25 (21), both of which are obtained by extrapolation, in one case to the first 24 minutes and in the other to zero time, in experiments in which the rate declined considerably with time. Our values, on the other hand, are all actual figures for the total of the 60-minute experiment. Other values obtained with rat brain slices range from 10.7 to 13.7 (13), while the same source gives one figure for minced rat brain of 5.3. Some higher values for minces were obtained by Elliott and Libet (21) with isotonic and hypotonic suspensions. Recalculation of some of their data gives  $Q_{\text{O}_2}$  values around 7.5 for isotonic and 2.3 for hypotonic suspensions. On the other hand, Colowick, Kalckar and Cori (23), with



reinforced extracts, obtained values (recalculated from their data) for  $Q_{O_2}$  between 3 and 9 approximately, which may be compared with our extract values of about 6 or 7. These authors give no figures for homogenates.

As has been pointed out on a previous occasion (1, 2), maximal enzyme activity is obtained by maximal dispersion of enzymes (cytolysis by distilled water during thorough homogenization) followed by maximal reinforcement with necessary co-factors. These procedures account for the higher activity of our preparations. When co-factors are added, cytolysis is obviously an advantage, since it eliminates as far as possible limitations due to diffusion. The result of avoiding cytolysis by the use of special osmotically adjusted saline mixtures when dealing with already damaged tissues is merely to complicate the picture by observing the behavior of an indeterminate mixture of cells, cell fragments, and free enzymes. Tissue slices, on the other hand, may perhaps give information of a different kind than homogenates if handled so as to avoid cell damage as far as possible, since factors of intracellular structure may become prominent. It is difficult to see, however, how one can be sure that no diffusion process is rate-limiting with slices; moreover, the thinner the slice, the greater is the proportion of damaged to intact cells.

From data such as those of Tables I and III, it is evident that the most important co-factors for respiration are the same as those for glycolysis: hydrogen acceptors, particularly DPN, and stable phosphate donors, such as HDP. These results are not very surprising; nor is it any more surprising that DPN seems even more critical for respiration than Cytochrome c—this is what one would expect on reflecting that the former is actively destroyed by damaged tissues, while the latter is merely diluted in the process of homogenization.

The most interesting result found with our active preparations is the occurrence of a high aerobic glycolysis in brain, so high as to indicate the total absence of a Pasteur effect. Since about 12 moles of glucose are glycolyzed for every mole oxidized, it seems plausible to assume that oxidation of pyruvate is a limiting factor in the system. This conclusion is strengthened by direct study of pyruvate oxidation in the brain homogenates.

Our data so far indicate a rapid aldolase and lactic dehydrogenase together with a very weak pyruvate oxidation system. Triose-phosphate oxidase may be more vigorous than the oxygen uptake shows, since under these conditions pyruvate competes with molecular oxygen

as a hydrogen acceptor. We have performed some preliminary studies of the triose-phosphate oxidase which are in agreement with this supposition.

It is not possible to give a definitive explanation of the absence of a Pasteur effect in brain, particularly since there is not yet available a definitive and widely accepted explanation of the nature of the Pasteur effect itself. We may point out, however, that previous work with brain has apparently placed glycolysis at a greater disadvantage than respiration, as is evident from the fact that our values for oxygen consumption are only three or four times higher than most of those previously obtained, whereas the rate of anaerobic glycolysis reported here and elsewhere (1, 2) is fifteen to twenty times greater than previous values.

As we have indicated above, DPN is certain to be absent in damaged brain tissues, while such factors as Cytochrome *c* and fumarate, being relatively stable, may be present in fair amounts in preparations (such as slices or concentrated pulps) which are not highly diluted. In a system with a negligible pyruvate oxidation and rapid lactic dehydrogenase, addition of sufficient amounts of DPN would tend to promote glycolysis even under aerobic conditions, since the dismutation between pyruvate and triose phosphate is independent of the rate of the cytochrome system; on the other hand, rapid pyruvate oxidation would not only remove substrate previously available for lactate formation, but also compete for DPN. The results of Adler and Calvet (24) on the ratio of oxidized to reduced DPN in aerobic and anaerobic yeast, if relevant to brain, do not contradict this hypothetical mechanism; the question at issue would seem to be the path taken by the reduced DPN rather than its proportion.

In tissues with a certain enzymatic makeup, then, DPN may act as an "inhibitor of the Pasteur effect." Whether this has any bearing on known inhibitors such as phenosafranine (25) is not obvious.

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#### SUMMARY

A method is presented for preparing tissue homogenates with high respiratory and glycolytic activity, and is applied to brain and liver.

For brain,  $Q_{O_2}$  values range between 15 and 20, with similar but slightly lower values for liver. Active mouse brain homogenates were prepared in the same manner.

Brain homogenates are found to have an extremely high aerobic glycolysis and virtually no Pasteur effect, manometric and chemical determinations being in agreement. About twelve moles of glucose are glycolyzed by the brain homogenate for every mole oxidized. The aerobic glycolysis and Pasteur effect in liver are more conventional.

The significance of these results is discussed.

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# On the Formation of Fatty Acids During the Retting of Flax\*

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## INTRODUCTION

In the industrial working of flax straw to obtain flax fiber, it is customary to allow the straw to ret in tepid water. The chief purpose of the retting is to free the fiber from the surrounding parenchymatous tissue. This liberation of the fibers is achieved through fermentation by certain anaerobic bacteria of pectin substances present in the parenchyma layer, which results in the loosening of this layer from the fiber. Since, however, the fiber also contains pectin, which is localized in the middle lamella between the fiber cells, the retting should not proceed further than to weaken this lamella to a slight degree. Such a mild attack on the lamella is also desirable from the viewpoint of spinning practice. If the retting is permitted to continue, the fiber tends to disintegrate into individual cells, thus losing its exceptional mechanical strength.

The above brief survey of the nature and purpose of flax retting indicates that it is valuable to know the details of the pectin fermentation which constitutes the principal factor in the retting process. As the chief products of the fermentation are the lower fatty acids, the chemical determination of these acids and of the pectin substances remaining in the flax straw offers an opportunity to obtain objective data regarding the progress of the retting. These data can then be correlated with the more subjective appraisal of the state of the flax fiber during the different phases of the process. For this purpose the

\* The research described in this paper is one of a series of investigations carried out at the request of the Flax Treatment Committee of the State Flax Board. The work was performed at the Institute of Biochemistry, Royal Technical University, Stockholm, and at the Institute of Physical Chemistry, University of Uppsala. The expenses were defrayed by funds from the State Council for Technical Research.

acids are more easily determined than the difficultly distinguishable pectin materials. In the latter case, indeed, it is necessary in practice to resort to purely arbitrary methods.

The pectin-fermenting bacteria can also attack substances of a carbohydrate nature other than pectin, *e.g.*, water-soluble carbohydrates, and convert these to acids, whereby an addition is made to the total quantity of acids which does not derive from the pectin. The same is the case when the dissolved carbohydrates are broken down by acid-forming, non-pectin-fermenting bacteria which occur in the spontaneous flora generated on the unsterilized flax straw. However, the greater part of the fatty acids formed in the warm-water retting of flax appear to be produced by the fermentation of pectin.

The five lowest fatty acids can all be formed in the retting process. Störmer (1) found acetic acid (HAc), butyric acid (HBu) and valeric acid (HVa), and Habermann (2) detected in addition formic acid (HFO). Lüdtke (3) also reported propionic acid (HPr).

In connection with an investigation of the microbiology of flax retting, one of us (Enebo) has made a number of experiments with regard to the fatty acid formation during the retting process. The present paper describes a selection of these experiments.

## EXPERIMENTAL

### (a) *Preparation of Flax*

Lightly retted flax was employed in all experiments. The roots and tops were removed from stalks of even thickness, which were then cut into pieces about 10 cm. long. These were bound together with cotton thread into bundles weighing about 60 g. Since the rettability of the flax straw is greatly diminished if it is subjected to sterilization by heat or chemicals, no such sterilization was performed. Consequently, no pure culture experiments with the straw were carried out, but all fermentations were brought about by the microflora of the straw itself.

### (b) *Preparation of Flax Pectin*

Five hundred g. of flax straw, cut into pieces about 0.5 cm. in length, was extracted for one hour in an enamel kettle with boiling acetone. The straw was filtered by suction, washed several times with acetone and frequently with water, and then allowed to stand for one hour with 0.5 *N* HCl. After suction filtration and thorough washing to remove HCl, the straw was treated with 5 l. of hot 0.5% ammonium oxalate solution. The mixture was allowed to stand for 30 minutes at about 80°C. The solution was removed by suction, filtered through kieselguhr, evaporated *in vacuo* to approximately 1 l. and precipitated with alcohol containing 12 ml. of conc. HCl/l., so that the final alcohol concentration was about 70%. The precipitate was washed

with 70% alcohol on a Jena G3 filter until the washings were free from chloride and oxalate. After further washing with hot 70% alcohol and finally with ether, the product was dried at 50°C. overnight. Yield,  $\pm 10$  g.

### (c) *Fermentation Procedure*

The rettings were carried out on different scales by two fundamentally similar methods. In the first case the retting was performed in preserving jars of 1.5 l. capacity containing 1200 ml. water or nutrient solution and 60 g. straw, pretreated as in (a). The jars were evacuated in the following manner. The air above the surface of the liquid was driven off with carbon dioxide, when the lid was replaced loosely. The jars were then placed in a vacuum desiccator which was evacuated. The tap was then rapidly removed, so that air flowed rapidly into the desiccator. The jars were then immediately closed before any air had time to enter. This simple method, which was proposed by Clausen (1930), is very suitable for anaerobic cultivations of different kinds. The last traces of oxygen may be eliminated by the insertion of a tube (externally sterile) containing pyrogallol and alkali.

When fermentation started, so much gas was usually developed in the jars that the lids were loosened. By fixing these with clamps, however, we were generally able to render the vessels so tight that no appreciable quantities of air entered. At this stage of the retting, moreover, the redox potential of the solution had reached its minimum value and was not affected by a very small introduction of air.

In the second case the retting was carried out on 100 times the above scale in an acid-resistant steel cylinder. In these experiments 6 kg. of whole flax stalks and 120 l. of water were employed. Air was not excluded: in retting on such a large scale rH decreases rapidly throughout the mass owing to the activity of the subsidiary flora which is always generated in spontaneous retting. The air absorbed at the surface of the liquid is consumed in the surface layer. The rettings on both the small and large scales were thus allowed to proceed under practically anaerobic conditions, which was of importance in view of the fact that certain of the acids formed are converted to carbon dioxide and water by bacterial action when the air supply is more generous.

The retting temperature was 35–37°C. The preserving jars were placed in a thermostat, while the large vessel was kept at constant temperature by means of a hot-water coil.

### (d) *Analytical Methods for the Lower Fatty Acids*

For the determination of the fatty acids formed in the retting process, the usual partition method was employed in a somewhat modified form, permitting a greater degree of accuracy than has hitherto been possible (*cf.* Osburn, Wood and Werkman (4)). In contrast to what appears to have been the practice of previous workers, we allowed for the effect of the molarity of the solution upon the partition coefficient.

A suitable quantity of retting liquor (up to 1000 ml.) was neutralized with NaOH against phenolphthalein and evaporated to about 10 ml. on the water bath. The sample was acidified with  $H_3PO_4$  and steam-distilled in a micro apparatus. Here the volume was further reduced, so that 100 ml. of distillate contained the whole quantity of acid. The acidity of the distillate was determined, after which it was diluted with water to 0.15 *N*. Fifty ml. of this acid solution was shaken in a separatory

funnel with 50 ml. of water-saturated isopropyl ether. The aqueous phase was titrated and the ether phase was shaken 4 times in succession with water saturated with isopropyl ether. The aqueous phase was titrated after each extraction. By these successive extractions HFo and HAc were removed from the ether phase which, after the third extraction, contained almost exclusively HPr, HBu and HVa. The quantities of the different acids were obtained from a specially constructed graph. As HFo and HAc have almost identical partition coefficients, it was convenient to determine only the sum of these acids in the extracts and then to determine HFo alone by oxidation with  $\text{HgCl}_2$ .

### (e) Retting Experiments

1. *Acid Formation in Spontaneous Retting without Previous Extraction of the Water-Soluble Components of the Straw.* In spontaneous retting, when the retting liquor is not changed, the acids formed will derive from water-soluble substances as well as from pectin. In addition to the pectin-decomposing bacteria, acid-forming bacteria of other types partake in these processes. Table I shows how pH, total

TABLE I  
*pH, Volatile Acids, Methanol and "A"- and "B"-Pectin during a Retting on the Semi-Industrial Scale*

Time	pH	Volatile acids as HAc	Methanol in retting liquor	"A"-pectin in straw	"B"-pectin in straw	Sum of "A"- and "B"-pectin
<i>hours</i>		<i>g./l.</i>	<i>mg./l.</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
0	—	—	—	2.70	1.72	4.42
8	4.99	0.78	10.0	2.46	2.00	4.46
16	4.86	0.93	18.0	2.18	1.31	3.49
24	4.66	1.44	26.0	1.57	1.06	2.63
32	4.51	1.89	58.0	1.08	0.99	2.07
41	4.51	2.22	75.0	0.95	1.01	1.96
48	4.47	2.37	62.5	0.99	0.64	1.63
56	4.44	2.41	68.5	0.90	0.45	1.35
64*	4.38	2.50	63.3	0.84	0.34	1.18
114	4.35	2.88	—	0.40	0.35	0.75

\* Retting completed.

volatile acids, methanol (according to von Fellenberg) and the contents of "A"- and "B"-pectin in the straw varied during a retting on the 120 l. scale (Lüdtke and Feiser (5), determination after Sandegren (6)).

Table II shows the relations between the volatile acids in the retting liquor in four different rettings on the 120 l. scale. In I, III and IV the analyses were made when the retting was complete, in II when it was only  $\frac{3}{4}$  complete.

TABLE II

*Relationship between the Volatile Acids in Some Spontaneous Rettings*

Retting	Percentage by weight				
	HFo	HAc	HPr	HBu	HVa
I	0.9	61.5	6.0	32.5	0.0
II	0.9	59.0	6.5	34.5	0.0
III	0.5	62.2	6.6	29.9	1.3
IV	0.6	62.2	6.7	29.9	1.2
Mean:	0.7	61.2	6.5	31.7	0.6

The relationships between the volatile acids at different times in the course of the same retting are shown in Table III.

TABLE III

*Relationship between the Volatile Acids during the Same Retting*

	Time in hours					
	20		48		72	
	g./l.	Per cent total acid content	g./l.	Per cent total acid content	g./l.	Per cent total acid content
HFo	0.021	4.4	0.014	0.9	0.017	0.8
HAc	0.265	55.2	1.004	60.9	1.284	58.9
HPr	0.023	4.8	0.121	7.3	0.157	7.2
HBu	0.154	32.1	0.510	30.9	0.722	33.1
HVa	0.017	3.5	0.00	0.0	0.00	0.0
Sum	0.480	100.0	1.649	100.0	2.180	100.0

The rettings described above were carried out on so large a scale that oxidative processes at the surface of the retting liquor presumably did not bring about any appreciable destruction of acids. Circumstances differ in small-scale retting, however, if atmospheric oxygen is freely admitted.



Table IV gives the analytical results from a retting on 5 l. scale in glass beakers with lids. In this case the straw was removed after 10 hours and the retting liquor was allowed to stand for several days without straw. The destruction of acids rapidly assumed priority, so that the pH rose and the directly titratable acid fell to a minimum.

TABLE IV  
*Destruction of Acids in Retting Liquor without Straw*

Time in hours	pH	Acid number <sup>*</sup>
10	5.54	2.75
22	5.18	3.26
34	5.58	0.81
46	6.02	0.70
58	6.43	0.08

\* Acid number = ml. 0.1 N alkali/50 ml. retting liquor for titration to pH 9.0.

2. *Acid Formation in Spontaneous Retting after Extraction of the Water-Soluble Components of the Straw.* Rettings with exchange of the retting liquor for the removal of water-soluble substances in the flax were carried out anaerobically in preserving jars at 35°C. After 24 hours the retting liquor was decanted off and water at 35°C. was added, after which the jars were allowed to stand for 45 minutes. The liquor was then replaced by new water which in its turn was immediately replaced by 1200 ml. of a nutrient salt solution having the following composition:

	<i>g./l.</i>
$(\text{NH}_4)_2\text{SO}_4$	5
$\text{KH}_2\text{PO}_4$	1
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	0.1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.3
$\text{NaCl}$	0.1
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.01

In some parallel experiments the ammonium sulphate was replaced by the same quantity of asparagine. All jars were provided with 6 g. of moist compressed yeast (to lower the redox potential). The air above the surface of the liquid was replaced by  $\text{CO}_2$  and the jars sealed to exclude air.

In the preliminary retting and the exchange of the retting liquor, by far the greater part of the water-soluble substances and their conver-

sion products were removed. The acids formed after the exchange of the liquid thus presumably derived largely from the substance unextractable with water but nevertheless fermentable, *i.e.*, especially the pectin substances, together with asparagine in the parallel experiments.

After 96 hours the flax was completely retted. About 1150 ml. of solution per jar could be decanted off.

When the acids were determined, the following values were obtained:

TABLE V

*Acid Formation by Extracted Flax Straw, with and without Addition of Asparagine*

	Without asparagine		With asparagine	
	<i>g./l.</i>	<i>Per cent</i>	<i>g./l.</i>	<i>Per cent</i>
HAc (HFO)	0.72	60.0	1.35	40.3
HPr	0.07	5.8	1.49	44.5
HBu	0.41	34.2	0.51	15.2
HVa	0.0	0.0	0.0	0.0
Sum	1.20	100.0	3.35	100.0

3. *Fermentation of Pure Flax Pectin with Granulobacter pectinovorum Beijerinck et van Delden and Clostridium felsineum (Carbone et Tombolato) Bergey et al.* The pectin was prepared by the procedure described under (b) above. The substrate consisted of 350 ml./jar of the above-mentioned nutrient salt solution (with  $(\text{NH}_4)_2\text{SO}_4$ ) containing 2% pectin and 1% cellulose (the latter as "carrier" for the bacteria). The nutrient solution was sterilized for one hour at 120°C. After the addition of the pectin, the complete substrate was steamed for 30 minutes. The adequacy of the sterilization was shown by the fact that a sample of the substrate showed no sign of fermentation when kept at 37°C.

TABLE VI

*Fermentation of Pure Flax Pectin with Gr. pectinovorum and Cl. felsineum*

	Amounts formed/100 g. pectin by:	
	<i>Gr. pectinovorum</i>	<i>Cl. felsineum</i>
	<i>g.</i>	<i>g.</i>
HFO	0.4	0.0
HAc	24.2	33.8
HPr	0.0	0.0
HBu	7.2	7.4
Total	31.8	41.2

The cooled medium was inoculated with 30 ml. of a seed culture of *Gr. pectinovorum* (carrot culture) or *Cl. felsineum* (potato culture). In addition, *Saccharomyces logos* was added from an agar slope. A tube with a solution of pyrogallol and caustic potash was placed in every jar, whereupon the jars were evacuated and sealed as before. The analyses were performed after the cultures had stood for 5 days at 37°C. The solid components of the mixtures were centrifuged off and treated with 0.5% ammonium oxalate solution. When alcohol was added to the oxalate solution there was no pectin precipitate but only an insignificant sediment.

## DISCUSSION

In the spontaneous retting of flax only acetic acid, propionic acid and butyric acid appear to be formed in large quantities, while formic acid and valeric acid appear in mere traces. Table II shows that the relative proportions of the three first-named acids at the conclusion of the retting are approximately constant in different rettings under the same experimental conditions. According to Table III these proportions are also nearly constant at different stages of one and the same retting. This indicates that the butyric acid formers, *i.e.*, especially the pectin-decomposing bacteria in the present case, begin to display their activity at a very early stage. A division of the retting process into a preliminary phase, during which the subsidiary flora is developed, and a main phase, when the pectin-decomposing bacteria attain their full development (Ruschmann 1923), thus appears to be unmotivated.

If the water-soluble components are removed and the extracted straw is then allowed to ret, approximately the same proportions between the acids are obtained as in the retting of unextracted straw, although the total acid content is naturally lower than in the latter case (Table V). It therefore appears that the pectin-decomposing bacteria convert pectin and soluble carbohydrates to the same products in the same proportions.

Addition of asparagine to extracted straw gave rise to a very large increase in the total acid content, the rise in propionic acid being especially marked (Table V). It therefore seems possible that, in normal rettings, the propionic acid is not derived from pectins or soluble carbohydrates but from amino acids, perhaps produced by the degradation of proteins. This hypothesis is supported by the fact that propionic acid is not formed in the fermentation of pure flax pectin with pure cultures of flax-retting bacteria.

In the fermentation of pure pectin, *Cl. felsineum* gave a larger quantity of acids than did *Gr. pectinovorum*. It is a remarkable fact

that the formation of butyric acid in these experiments was proportionally smaller than in the fermentation of flax straw.

### SUMMARY

The formation of lower fatty acids in the warm-water retting of flax and flax pectin under different conditions has been investigated. Only acetic acid, propionic acid and butyric acid were formed in appreciable quantities in the spontaneous retting of flax, while formic acid and valeric acid appeared only as traces. The fermentation of pure flax pectin with pure cultures of anaerobic flax-retting bacteria gave only acetic acid and butyric acid.

The proportions between the acids were approximately constant both in different rettings under the same conditions and at different phases of the same retting. Acetic acid formed about 61%, propionic acid 6-7% and butyric acid about 32% of the total acid content.

In the retting of flax straw previously extracted with water the proportions of the acids were nearly the same as in retting without preliminary extraction. The pectin and the extracted substance are thus fermented to the same products in the same proportions.

Addition of asparagine to water-extracted flax straw gave rise to a considerable increase in the total amount of fatty acids formed in the retting, propionic acid showing an especially marked increase. It therefore appears possible that, in normal retting, propionic acid is formed not from compounds of the carbohydrate type but from amino acids, especially in view of the fact that pure flax pectin yielded no propionic acid.

*Clostridium felsineum* formed larger quantities of acids from pure flax pectin than did *Granulobacter pectinovorum*. The formation of butyric acid in relation to that of acetic acid was proportionally less in this case than in the fermentation of flax straw.

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## Purified Rations and the Requirement of Folic Acid for Foxes \*

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### INTRODUCTION

When thiamine was found to be effective in preventing Chastek paralysis in foxes the question naturally arose concerning the importance of other members of the B complex in the nutrition of this species. Studies on this group of vitamins have been greatly simplified by the use of purified rations. Thus, a similar experiment to that which we reported for mink (1) was started using adult foxes, and later weanling fox pups. Morgan *et al.* (2) reported the use of a partially purified ration for fox pups in studies on the anti-grey hair factor; however, in their experiments, the pups were fed a ration devoid of meat for only a short period. In this paper we wish to present data on the use of purified rations and the requirement of folic acid for adult and young foxes.

### EXPERIMENTAL

Six adult silver foxes from 5 different litters, and 9 weanling silver, platinum silver, and red fox pups from 4 different litters were used in these studies. Previous to being placed on experiment the adult foxes were fed a ration containing 55% horsemeat, (including bone), 7% fresh liver, 32% cereal mixture, and 6% tomato puree. The pups were weaned at 6-7 weeks of age and placed directly on experiment. They were treated twice at weekly intervals with tetrachlorethylene capsules for possible worm infection, dusted with DDT for external parasites and toe tendons were clipped to prevent excess clawing or scratching. All animals were housed in individual outdoor pens with raised wire floors. The purified rations used were the same as those previously described for mink (1). The basal ration consisted of sucrose 66%, casein (Smaco, Vit. Test) 19%, cottonseed oil 8%, cod liver oil 3%, and salt mixture 4%.

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Each 100 g. of ration was supplemented with 200  $\gamma$  thiamine chloride, 200  $\gamma$  pyridoxine hydrochloride, 400  $\gamma$  riboflavin, 1.5 mg. calcium pantothenate, 4 mg. niacin, and 100 mg. choline chloride. Where indicated, some animals received in addition, 100  $\gamma$  folic acid and 50  $\gamma$  biotin/100 g. ration and daily supplements of 20 mg. inositol, 50 mg. *p*-aminobenzoic acid, 2 mg.  $\alpha$ -tocopherol, and 0.5 mg. 2-methyl-1,4-naphthoquinone. Weekly weight and daily food consumption records were taken. Three cc. blood samples were collected from the radial vein at selected intervals. Hemoglobin determinations were done according to the method of Evelyn (3); hematocrit readings were obtained with Wintrobe tubes. White and red blood cell counts and white differential counts were determined by the usual methods.

Adult foxes and weanling pups began eating the purified ration within 2 days. The average daily food consumption for adult foxes and pups was approximately 30 and 50 g./kg. of body weight, respectively.

### *Studies with Adult Foxes*

Four adult foxes (Nos. 35, 38, 39, 40) received the basal ration (19% casein), one animal (No. 36) the basal plus gelatin (10% gelatin replacing sucrose), and one animal (No. 37) the high protein ration (30% casein). After receiving the various purified rations for 19–24 weeks the total loss in body weight varied from 1,150 to 1,940 g. Weight curves for animals 35, 36, and 37 are shown in Fig. 1. This weight loss was accompanied by a decrease in hemoglobin and red and white

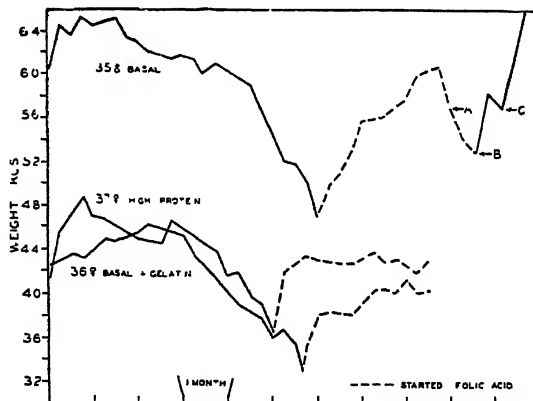


FIG. 1. Body Weight Curves of Foxes 35, 36, 37. Folic acid therapy 0.5 mg. per day. A, started biotin, *p*-aminobenzoic acid, inositol,  $\alpha$ -tocopherol, 2 methyl-1,4-naphthoquinone, and 10 mg. ascorbic acid per daily supplement. B, fed 400 g. of fresh beef liver over period of 1 week. C, started supplementation of the ration with 10% fresh beef liver.

blood cell counts. The blood analyses of two typical foxes are given in Table I. Anorexia was evident during 3 to 4 days prior to therapy. Folic acid was fed at levels varying from 25 $\gamma$ /100 g. ration to 0.5 mg./daily feeding. Anorexia disappeared within 2 days and body weight, hemoglobin concentration and red and white blood cell counts increased rapidly.

Eleven weeks after folic acid therapy had been started in fox 35, a rapid loss in body weight occurred. Biotin, *p*-aminobenzoic acid, inositol, vitamins E, K, and C supplementation was ineffective. The feeding of 400 g. fresh beef liver over a period of one week produced a rapid gain in weight. When the liver was removed a loss in body weight again was noted. Ten *per cent* fresh liver was then included daily in the ration. In a period of 6 weeks body weight increased 3 kg., hemoglobin rose from 15.1 to 18.2 g.-%. The typical reversal of the percentage of neutrophils and lymphocytes similar to that noted in the monkey (4) was corrected.

It was of interest to determine whether folic acid-deficient foxes could utilize folic acid conjugate as present in yeast. Thus, after fox 39 had lost 1 kg. body weight and the hemoglobin level was reduced to 9.8 g.%, 5 g. yeast was fed orally, and 1% yeast added daily to the ration. Bacteriological assay of the yeast by the method of Snell (5) and Luckey *et al.* (6) using *S. faecalis* and *L. casei* organisms yielded 30 and 32  $\gamma$  folic acid/g., respectively, when the yeast was digested with frozen water extract of hog kidney as described by Bird *et al.* (7). Before digestion with hog kidney enzyme the yeast assayed 0.8  $\gamma$ /g. with *S. faecalis* and 3.9  $\gamma$  with *L. casei*, indicating that the greater part of the folic acid occurred as folic acid conjugate. During 2 weeks of such therapy there was an additional loss in body weight and continued lowering of hemoglobin concentration and red blood cell count. The animal was extremely weak, therefore 2 mg. folic acid was fed orally in addition to supplementing the ratio daily with 25  $\gamma$ /100 g. Ten weeks of this therapy produced an increase in body weight from 3.5 kg. to 6.5 kg., hemoglobin from 8.01 to 15.48 g.-%, red blood cells from 4.47 to 10.69 million/mm.<sup>3</sup>, and white blood cells from 5,000 to 7,900/mm.<sup>3</sup>

Fox 40, after 23 weeks on the basal ration, lost 1,350 g. body weight, and hemoglobin was reduced from 15.68 to 9.8 g./100 cc. blood. At this time food consumption was approximately 75% normal. Two *per cent* yeast was added to the ration for a period of 3 weeks. Loss of body



weight and reduction in hemoglobin and red blood cell counts continued during this period. A water extract of yeast hydrolyzed with hog kidney enzyme was fed at a level equivalent to 1% yeast. After 2 weeks of such therapy, hemoglobin was increased from 7.00 to 11.55 g.-% and red blood cell counts from 6.6 to 8.24 million/mm.<sup>3</sup>

### *Studies with Fox Pups*

Selected fox pups from 2 platinum silver litters, a silver litter and 1 red pup were placed on experiment. A total of 6 pups (Nos. 3, 8, 9, 10, 15, and 27) were fed the basal ration. Nos. 4 and 22 received the basal ration supplemented with 100  $\gamma$  folic acid, No. 7 received the

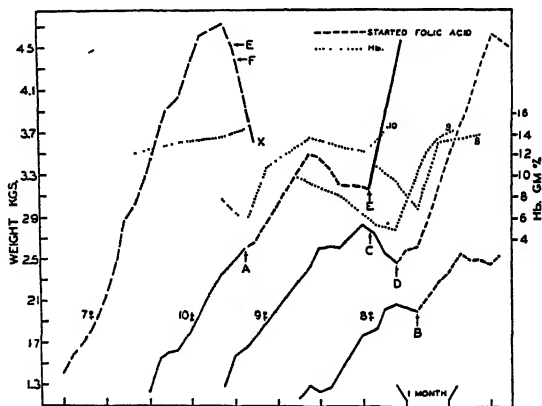


Fig. 2. Growth Curves and Hemoglobin Values of 4 Littermate Silver Fox Pups. Nos. 8, 9, and 10 were started on the basal ration. No. 7 received the basal supplemented with 100  $\gamma$  folic acid and 50  $\gamma$  biotin/100 g. ration. A, started 50  $\gamma$  folic acid/100 g. ration. B, fed orally 250  $\gamma$  folic acid and started 25  $\gamma$ /100 g. ration. C, 2% yeast supplement. D, started 25  $\gamma$  folic acid/100 g. ration. E (pup 7), started *p*-aminobenzoic acid, inositol,  $\alpha$ -tocopherol, and 2-methyl-1, 4-naphthoquinone. F, fed orally *via* capsule 700 mg. inositol, 550 mg. *p*-aminobenzoic acid, and 650 mg. ascorbic acid. E (pup 10), started 10% fresh liver.

basal plus 100  $\gamma$  folic acid and 50  $\gamma$  biotin/100 g. ration, and No. 26 received the basal supplemented with 3% Wilson's whole liver powder.

Growth curves and hemoglobin levels of littermate silver fox pups, Nos. 7, 8, 9 and 10 are shown in Fig. 2. After 9-13 weeks on the ration, pups 8, 9, and 10 exhibited an anemia characterized by a hemoglobin concentration of 4.97 to 6.87 g.-%, red blood cell counts of 2.65

to 4.8 million/mm.<sup>3</sup> and white blood cell counts of 3,900 to 6,050/mm.<sup>3</sup> Two *per cent* yeast was added to the ration for pup 9 for a period of 2 weeks with no alleviation of the anemia or increase in body weight. Folic acid was fed at levels varying from 25 to 100  $\gamma$ /100 g. of ration. Two weeks after therapy was initiated hemoglobin and red blood cell counts had increased over 100% over the anemic levels. Pup 7, which received folic acid and biotin from the start of the experiment, gained weight at a rapid rate for 15 weeks. Hemoglobin level, red blood cell, and white blood cell counts were normal. Then, daily food consumption began to decrease with a subsequent loss in body weight. When the first loss in weight was observed, vitamins E, K, *p*-aminobenzoic

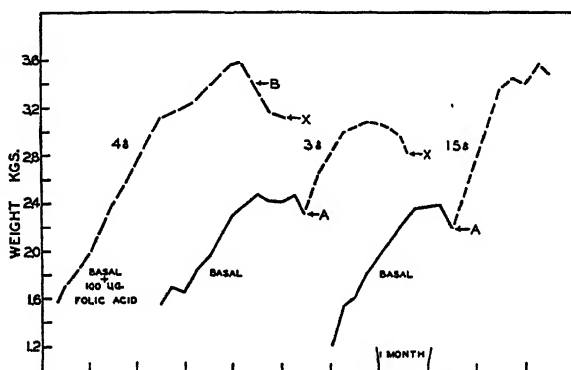


FIG. 3. Growth of Platinum Silver Fox Pups 3 and 4 and Red Fox Pup 15. A, 100  $\gamma$  folic acid orally and started 25  $\gamma$ /100 g. ration. B, 250  $\gamma$  biotin fed orally and started daily supplement of 50  $\gamma$ /100 g. ration and also started *p*-aminobenzoic acid, inositol,  $\alpha$ -tocopherol, and 2-methyl-1,4-naphthoquinone. X, died.

acid and inositol were added to the ration. Body weight continued to drop and, at the 17th week, 700 mg. inositol, 550 mg. *p*-aminobenzoic acid, and 650 mg. ascorbic acid were fed orally *via* capsule. Anorexia continued, with a critical loss in body weight. Fresh beef liver was offered, but the animal refused it. General weakness and chewing of fur was noted. The animal died after being on experiment for 17 weeks and 5 days. Autopsy revealed fatty degeneration of the liver. Gross appearance of the other organs and tissues was normal.

Growth curves of platinum silver littermate pups, Nos. 3 and 4, and red pup No. 15 are shown in Fig. 3, blood analyses are given in Table I. At 10–12 weeks on experiment pups 3 and 15 exhibited the

TABLE I

Fox No.	Days on expt.	Body wt. Kg.	Hb.	Hematocrit	RBC	WBC
			g-%	Per cent	millions/mm. <sup>3</sup>	thousands/mm. <sup>3</sup>
Basal Ration						
39 ♂	148	4.40	9.8	—	6.57	9.0
	148	(Started 1% yeast)				
	155	3.49	8.01	—	4.47	5.0
	155	(Fed 2 mg. folic acid orally. Started 25 γ folic acid/100 g. ration)				
	169	4.14	9.88	26	6.31	6.15
	195	5.23	13.5	45	10.69	6.6
High Protein Ration						
37 ♀	135	3.63	10.4	28.1	—	8.10
	135	(Started 500 γ folic acid per day)				
	168	4.30	17.04	46.1	14.91	11.15
Basal Ration						
3 ♂	49	2.38	8.36	24	5.24	8.75
	84	2.35	5.80	17.2	3.41	4.65
	84	(Fed 100 γ folic acid orally—started 25 γ/100 g. ration)				
	92	2.65	9.96	29	4.46	4.8
	119	3.07	12.21	33	6.21	6.91
	149	2.84	7.24	19.6	7.42	8.85
	152	died				
Started on Basal Ration + 100 γ Folic Acid/100 g. Ration						
4 ♂	49	2.76	10.25	32	5.94	15.45
	113	3.57	11.37	36	6.86	14.75
	133	3.40	9.22	23.2	7.22	17.70
	133	died				
Basal Ration						
15 ♂	48	2.36	10.15	29	6.77	8.30
	70	2.21	7.87	22.8	4.62	6.40
	70	(Fed 100 γ folic acid orally, started 25 γ/100 g. ration)				
	82	2.73	12.14	36.0	9.26	6.7
	126	3.49	15.19	47.0	12.0	11.75
Started on Basal Ration + 3% Whole Liver Powder						
26 ♀	49	2.95	12.56	36	8.82	10.65
	112	3.76	14.67	44.4	9.54	10.25

typical anemia previously described. An initial dose of 100  $\gamma$  folic acid was fed orally in addition to adding 25  $\gamma$ /100 g. of ration. Response in growth and alleviation of the anemia were observed. Pup 3 gained weight for 5 weeks after starting folic acid therapy and then plateaued in body weight. Pup 4 continued to gain weight for 16 weeks. In both animals, as the first loss in body weight was noted, the ration was supplemented with biotin, *p*-aminobenzoic acid, inositol,  $\alpha$ -tocopherol, and 2-methyl-1,4-naphthoquinone. The addition of these vitamins failed to prevent further loss in body weight and death resulted suddenly.

Littermate platinum silver pups Nos. 22, 26, and 27 afforded a comparison among pups receiving the basal (No. 27), the basal plus folic acid (No. 22), and the basal plus 3% whole liver powder (No. 26). After 5 weeks on experiment, hemoglobin concentration of pup 27 was 7.66 g./100 cc. of blood in comparison to 11.71 and 12.06 for pups Nos. 22 and 26, respectively. At 8 A.M. on the morning of the 7th weekly weighing, No. 27 appeared normal, but at 11:30 was found dead in the cage. Fatty degeneration of the liver and congestion of the coronary vessels were noted upon autopsy. The gross appearance of the other tissues and organs was normal. The growth of pups 22 and 26 was at virtually the same rate for 16 weeks. Pup 22 then began to lose weight at a rate similar to that observed in pups 4 and 7, whereas No. 26 continued to grow at a rate comparable to pups receiving the stock meat, vegetable and cereal ration.

### DISCUSSION

Although the fox is taxonomically related to the dog, the requirements of folic acid, and also another factor in liver, differ greatly in the two species.

The 6 adult foxes placed on purified rations of varying levels of protein developed deficiency symptoms in 19–24 weeks. Loss in body weight varied from 1.12 to 1.94 kg. Hemoglobin was reduced in 5 cases, varying in individuals from 7.0 to 13.11 g./100 cc. of blood. Red blood cell counts determined on 4 animals at the time just prior to therapy ranged from 4.47 to 10.27 million/mm.<sup>3</sup> White blood cell counts varied from 4.6 to 8.45 thousand/mm.<sup>3</sup> The administration of folic acid at levels from 25  $\gamma$ /100 g. ration to 500  $\gamma$  per daily feeding resulted in a dramatic recovery from anorexia, body weight loss and anemia.

The 6 fox pups placed on the purified basal ration developed critical deficiency symptoms in 7 to 14 weeks. Loss in body weight seemed secondary to the onset of anemia. Hemoglobin was reduced to values varying from 4.97 to 8.87 g.-%; whereas the hemoglobin concentration

of control animals receiving folic acid ranged from 11.63 to 13.77 g.-%. Red blood cell counts were reduced to 2.65–4.80 million/mm.<sup>3</sup> as compared to 8.18–9.36 million/mm.<sup>3</sup> for the controls. White blood cell counts were reduced to 3,900–6,400/mm.<sup>3</sup> as compared to 7,200–14,300/mm.<sup>3</sup> for controls. The administration of folic acid at varying levels of 25  $\gamma$  to 100  $\gamma$ /100 g. ration resulted in a dramatic increase in hemoglobin, red and white blood cell counts.

Yeast containing 30–32  $\gamma$  of folic acid conjugate/g. when fed at levels of 1 or 2%, to folic acid-deficient animals, failed to correct the anemia or loss in body weight. A water extract of yeast hydrolyzed with kidney enzyme when fed at an equivalent level of 1% yeast, produced the typical increase in hemoglobin and red blood cells as obtained with synthetic folic acid.

Preliminary observations on 4 fox pups and 1 adult fox at this time indicate that our purified basal ration supplemented with folic acid or folic acid and biotin produced a deficiency characterized by loss in body weight, anemia, and death in periods of 15 to 36 weeks respectively. Supplementation of the ration with *p*-aminobenzoic acid, inositol, vitamins E, K, and C, failed to alleviate this loss in body weight. The feeding of fresh beef liver to animals 10 and 35 produced an immediate gain in body weight and alleviation of the anemia. The requirement of the fox for folic acid and an additional liver factor closely parallels the observations reported for the monkey (4) and the mink (1).

The use of purified rations for foxes presents a useful tool in studying the nutritional requirements of the B complex for this species. Growth of pups receiving the basal ration supplemented with folic acid for a period of 15 to 17 weeks compares favorably with pups fed a meat, cereal, and vegetable ration. The requirement of fox pups for riboflavin, pyridoxine, niacin, and pantothenic acid has been demonstrated (unpublished data). These studies have indicated the acute need for some of the B complex vitamins and also emphasized the speed of onset of deficiency symptoms.

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## SUMMARY

Purified rations were successfully employed in studying the nutritional requirements of adult foxes and fox pups for various members of the vitamin B complex.

Foxes receiving highly purified vitamin B complex-free rations supplemented with thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, and choline develop a deficiency characterized by anemia (low hemoglobin levels, low red and white blood cell counts), loss in weight, anorexia and death.

The administration of folic acid to 6 adult foxes and 5 pups at levels varying from 25  $\gamma$ /100 g. of ration to 500  $\gamma$  per daily feeding at the time of deficiency symptoms resulted in immediate recovery. The rapid regeneration of hemoglobin and red blood cells emphasizes the hematopoietic role of folic acid.

Folic acid conjugate, as present in yeast, was not utilized when fed to folic acid-deficient foxes at levels corresponding to synthetic folic acid, which produced a rapid remission of symptoms. A water extract of yeast hydrolyzed with kidney enzyme produced a remission of the anemia.

Preliminary observation indicates that fox pups and adults receiving all the known vitamins in crystalline form still develop a deficiency characterized by anorexia, loss in body weight, anemia, and death. The feeding of fresh liver resulted in a rapid increase in body weight and remission of the anemia. This suggests that fresh liver contains an unknown factor necessary for normal nutrition of foxes.

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# A Comparison of Plasma Protein Concentration, Hemoglobin and Hematocrit Values Determined by Chemical Methods and Calculated from Specific Gravity \*

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## INTRODUCTION

A direct relationship between specific gravity and protein concentration of plasma has been demonstrated by Moore and Van Slyke (1) and these findings were later confirmed by Weech, Reeves and Goettsch (2). Since the methods have been simplified (3, 4, 5, 6) there has been an increased interest in the clinical application of the specific gravity determinations. In connection with a project on metabolic alterations in experimental burns in dogs, the plasma protein, hemoglobin and hematocrit changes were studied. This afforded an opportunity to compare results obtained by the falling drop and copper sulfate specific gravity methods with the chemical values for these determinations.

## *Experimental*

Fasting, non-hemolyzed samples of blood and plasma were obtained from preburn and postburn dogs. The first samples from the postburn dogs were obtained on the fourth day following the burn so that none were taken from animals during the period of shock.

Plasma protein was determined by the micro-Kjeldahl method (7). Theoxy hemoglobin method described by Evelyn (8) was used for the determination of hemoglobin. The "K" value was calculated from the oxygen capacity of several samples of dog

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blood. The factor 1.36 cc. of oxygen/g. of hemoglobin, as determined by Bernhart and Skeggs (9) was used in the calculation. Hematocrit determinations were made with the use of Sanford-Magath cell volume tubes, centrifugation being carried out at 3000 r.p.m. for 45 minutes. The specific gravity of plasma was determined by the falling drop method as described by Barbour and Hamilton (3) and the copper sulfate method described by Phillips, Van Slyke *et al.* (5). The latter method was also used for obtaining specific gravity of whole blood. Heparin was employed as the anti-coagulant.

## RESULTS

### *Specific Gravity and Protein Concentration*

Figs. 1 and 2 show the specific gravity of plasma determined by the falling drop and copper sulfate methods respectively, plotted against protein concentration determined by the micro-Kjeldahl method. The

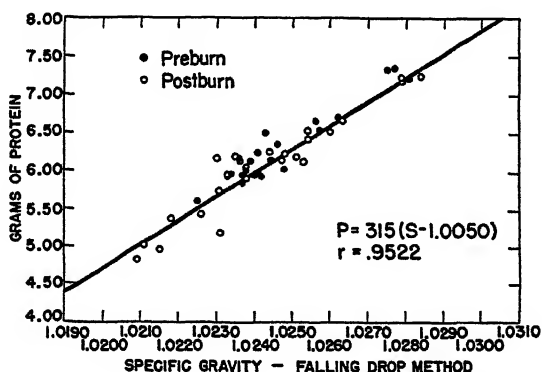


FIG. 1. Concentration of Protein (Kjeldahl) of Dog Plasma Plotted Against Specific Gravity.

correlation coefficient and regression line with standard error of estimate (10) for each method is given below.

Falling drop method  $r = 0.9522$

$P = 315 (S = 1.0050) \pm 0.12$

Copper sulfate method  $r = 0.9061$

$P = 325 (S = 1.0059) \pm 0.24$

where  $r$  = the correlation coefficient,  $P$  = protein concentration, and  $S$  = specific gravity of the plasma. Both methods show a highly significant correlation between specific gravity of plasma and protein

concentration. (The data from the preburn and postburn samples were handled separately, but since there was no significant difference between the two groups the figures were combined.) Weech, Reeves and Goettsch (2), using the pycnometer for determining specific gravity of dog plasma found a higher correlation, namely, 0.991532. Adams and Ballou (11), using the copper sulfate specific gravity method in a group of patients report a correlation coefficient of 0.74. Several investigators report a good agreement between protein values determined by the specific gravity and Kjeldahl methods, but do not give correlation values. In contrast to these findings, Zozaya (12) and Looney (13) did not find a satisfactory relationship between the two methods.

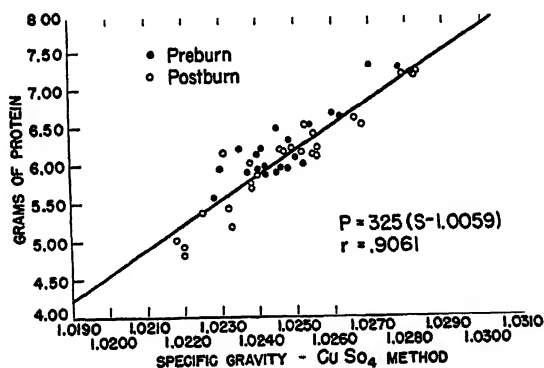


FIG. 2. Concentration of Protein (Kjeldahl) of Dog Plasma Plotted Against Specific Gravity.

Table I compares the calculated protein concentration obtained from the falling drop and copper sulfate specific gravity methods with the Kjeldahl values. With the first method 77% of the cases agreed within 0.2 g. with the Kjeldahl value, and with the copper sulfate method 68%. There was statistically no significant difference between the two specific gravity methods. The mean value obtained by the falling drop method was 1.0245 with a standard deviation of 0.0018 and for the copper sulfate method 1.0248 with a standard deviation of 0.0016. The mean Kjeldahl protein concentration for the group (preburn and postburn) was 6.16 g./100 cc. of plasma with a standard deviation of 0.58 g.

TABLE I  
*Comparison of Protein Concentration Calculated from Specific Gravity  
 and Determined by the Kjeldahl Method*

Difference between total protein by micro- Kjeldahl and specific gravity methods	Number of instances			
	Falling drop method <sup>1</sup>	Equation reported by Weech <i>et al.</i> <sup>2</sup>	CuSO <sub>4</sub> method <sup>3</sup>	Equation reported by Weech <i>et al.</i> <sup>3</sup>
<i>g.</i>				
±0 — ±0.10	23	13	15	17
±0.11 ±0.20	13	13	17	13
±0.21 ±0.30	7	9	7	9
±0.31 ±0.40	2	6	3	2
±0.41 ±0.50	1	3	4	3
±0.51 ±0.60	1	3	1	2
±0.61 ±0.70				1
±0.71 ±0.80				
±0.81 ±0.90				
±0.91 ±1.00				

<sup>1</sup> *P* = 315 (*S*-1.0050).<sup>2</sup> *P* = 340 (*S*-1.0069).<sup>3</sup> *P* = 325 (*S*-1.0059).

There are various equations in the literature for calculating protein from specific gravity. There is insufficient evidence at present, however, to give preference to any one of these equations, or that there is a species difference in the relationship. Table I compares the calculated values using the equation reported by Weech, Reeves and Goettsch (2), which was done on dog plasma, with the equations obtained from the present study. There is excellent agreement with the equation obtained from the copper sulfate specific gravity values, but not quite as good with the equation obtained by the falling drop specific gravity method.

### *Specific Gravity and Hematocrit Values*

The formula of Ashworth and Tigertt (14) was used for the calculation of cell gravity and hematocrit. These same authors report a mean specific gravity of red cells for dogs of 1.0953 with a standard deviation of 0.00205. The cell specific gravity in the present series of normal dogs was 1.0960 with a standard deviation of 0.00158. The latter factor was used in the calculation of the hematocrit values.

The average difference between calculated and determined values was 1.11 cc. of cells/100 cc. of blood, with a standard deviation of difference of 1.07 and standard error of difference of 1.46. The number of cases that fell within designated differences are shown in Table II. The greatest difference in the preburn group was 2.2 cc. of cells/100 cc. of blood.

TABLE II

*Comparison of Hematocrit Values Calculated from Specific Gravity  
and Determined by Centrifugation*

Difference in cc. of cells/100 cc. of blood	Number of instances
$\pm 0.1-0.5$	16
$\pm 0.6-1.0$	7
$\pm 1.1-1.5$	9
$\pm 1.6-2.0$	9
$\pm 2.1-2.5$	3
$\pm 2.6-3.0$	1
$\pm 3.1-3.5$	1
$\pm 3.6-4.0$	1

*Specific Gravity and Hemoglobin Values*

Hemoglobin was calculated from the formula suggested by Phillips, Van Slyke *et al.* (5). It was found that the hemoglobin was 2.4 g. lower/100 cc. of cells in the present series of normal dogs than the value obtained in humans by the aforementioned group. Our value, as well as the cell specific gravity of 1.0960, was substituted in their equation for the calculation of the hemoglobin content per 100 cc. of blood.

The average difference between determined and calculated hemoglobin per 100 cc. of blood was 0.28 g., with a standard deviation of difference of 0.25 and standard error of difference of 0.36 g. The average specific gravity of plasma in the group of normal dogs was 1.0248. When hemoglobin was calculated from specific gravity of whole blood alone, assuming a normal specific gravity of plasma, the average difference between calculated and determined hemoglobin was 0.43 g., with a standard

TABLE III

*Comparison of Hemoglobin Values Calculated from Specific Gravity  
and Determined Colorimetrically*

Difference g.	Use of specific gravity of blood and plasma	Use of specific gravity of blood alone
$\pm 0.0$	5	7
$\pm 0.1$	12	5
$\pm 0.2$	10	5
$\pm 0.3$	7	6
$\pm 0.4$	5	6
$\pm 0.5$	1	4
$\pm 0.6$	3	2
$\pm 0.7$	0	3
$\pm 0.8$	2	0
$\pm 0.9$	2	3
$\pm 1.0$	0	4
$\pm 1.1$	0	1
$\pm 1.3$	0	1

deviation of difference of 0.39 and standard error of difference of 0.55. Thus, 95% of the hemoglobin concentrations calculated from the specific gravity of blood and plasma should come within two standard errors, or 0.7 g., and using blood alone, within 1.1 g. (15). Table III shows the number of cases, determined by each method, that fell within designated differences, and shows that, although the specific gravity of plasma and blood gives greater accuracy, the specific gravity of whole blood alone gives results which may be of clinical value.

### DISCUSSION

The results confirm the reports (4, 5, 16, 17, 18, 19, 20) that the simplified methods for determining specific gravity give clinically fairly satisfactory results for protein concentration as well as hemoglobin and hematocrit values. No attempt was made to explain the discrepancies between the specific gravity values and the protein concentration. The cases were not confined to the postburn group. An elevated non-protein nitrogen was present in only one instance. Changes in albumin-globulin ratio appeared to have no effect on the calculated protein concentration. This finding has also been noted by Atchley and Bacon (16) and by Nugent and Towle (21). However, Adams and Ballou (11) felt that an inverse relationship seemed to affect the results. Simeone and Sarris (17) discuss the error that may occur from high glucose concentration. Kagan (4) found that moderate elevations in cholesterol content did not significantly affect the specific gravity-calculated protein values. Cole, Allison and Boyden (22) report that the specific gravity-protein concentration relationship is very poor in conditions of shock.

Although the calculated hemoglobin and hematocrit values do not check with the determined values as closely as the figures reported by Phillips, Van Slyke *et al.* (5) they are within the range of differences given in other reports (16, 19). The greatest differences, in the present series of cases, were found in the postburn group. A discussion of the error, and review of work on pathological bloods is brought out in the report by the above workers.

There have been reports (14, 23, 24) suggesting that the specific gravity method is a convenient method of estimating changes in the blood volume and the state of hydration or nutrition. Thus, such determinations have been advocated as a guide to the necessary treatment. Although the method is of some value, the interpretation of such data for treatment requires the same care as is applicable to any chemical method (5). It has been demonstrated that in some instances

changes in hematocrit, hemoglobin and plasma protein concentration are of limited significance in determining the patient's state of hydration or nutrition (25, 26). The specific gravity methods, therefore, seem sufficiently accurate, in many cases, to give the desired information. However, it should be emphasized that such findings should be interpreted only as they are related to the history and clinical findings.

### SUMMARY

The specific gravity of 47 samples of dog plasma was determined by the falling drop and copper sulfate methods. There was no significant difference between the two methods and both showed a highly significant correlation when plotted against protein concentration. With the first method 77% of the cases, and the latter method, 68% of the cases agreed within 0.2 g. of the Kjeldahl value.

The specific gravity of whole blood was also determined by the copper sulfate method to calculate hemoglobin and hematocrit. The average difference between the determined hemoglobin and the calculated value from specific gravity of blood and plasma was 0.28 g., and from specific gravity of blood alone 0.43 g. The average difference between calculated hematocrit and centrifuged value was 1.11 cc. of cells/100 cc. of blood.

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# Trypsin Inhibitor. III. Determination and Heat Destruction of the Trypsin Inhibitor of Soybeans\*

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## INTRODUCTION

The announcement by Ham and Sandstedt (1) of a trypsin inhibitor in unheated soybeans and the demonstration of Ham, Sandstedt and Mussehl (2) that the feeding of the trypsin inhibitor decreases the growth rate of chicks has opened a new phase of investigation on the nutritive value of the soybean. It is well known that moist heat treatment of soybeans improves their nutritive value. Caskey and Knapp (3) have proposed a urease test for checking adequacy of heat treatment of soy. Since the destruction of the trypsin inhibitor appears to be the primary concern in heat treatment of soybean, investigations made to determine the degree of destruction of the inhibitor by heat are reported in this study.

To measure the destruction of the inhibitor by heat, or the amount of inhibitor used in feeding trials, as well as determination of the purity of various preparations of inhibitor, it was necessary to devise a quantitative measure of the inhibitor. Qualitative procedures for demonstrating trypsin inhibition by decreased trypsin action have been used by Northrop (4), Balls and Swenson (5), Ham *et al.* (1, 2) and Bowman (6). Wunderly (7) and Grob (8) proposed quantitative methods for expressing the trypsin inhibitor present in blood serum. For a proper presentation of an inhibitor unit, several factors which may affect *in vitro* trypsin digestion in the presence of the inhibitor were investigated in this study.

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## METHOD

The method of Anson (9) for trypsin assay was used throughout these studies. As carried out in this laboratory, 1 ml. of enzyme was incubated for 5 minutes at 37°C. with 5 ml. of 2.2% hemoglobin substrate at pH 7.5. Ten ml. of 0.3 *N* trichloroacetic acid were added to precipitate undigested protein and an aliquot of the filtrate used for color development with the diluted phenol reagent of Folin and Ciocalteu (10). Color intensity was read on a Model 11 Coleman spectrophotometer and the readings expressed as tyrosine by reference to a standard curve. The initial chromogenic value, expressed as tyrosine, was obtained by similar procedure, except that the trichloroacetic acid was added immediately without incubation. The chromogenic substances, expressed as tyrosine, liberated due to the action of the trypsin were obtained by difference and units of trypsin were then calculated from the liberated tyrosine value. Smaller quantities of trypsin were required by this procedure than were required for the visual colorimeter. The inhibitor was incorporated into the procedure by mixing with the trypsin prior to addition to the substrate. To accomplish this, a stock trypsin solution approximately ten times stronger than required was prepared. Ordinarily 1 ml. of this stock solution was mixed with an aliquot of an acid extract of soybean meal (adjusted to pH 7.5) and then diluted to 10 ml. This was designated as the enzyme solution and 1 ml. added to the substrate.

## EXPERIMENTAL

*Influence of Variations in Concentration of Substrate, Trypsin and Inhibitor on the Inhibition effect*

In a study of the effect of the soybean trypsin inhibitor on the digestion of hemoglobin by trypsin, 3 variables were investigated. In Table I are recorded the results of variation of the concentration of hemoglobin with constant trypsin and inhibitor. The extent of digestion, both with and without the inhibitor, as well as the extent of inhibition,

TABLE I  
*Inhibition with a Constant Amount of Trypsin and Inhibitor with Various Amounts of Hemoglobin*

ML stock trypsin/ml. enzyme soln.	ML inhibitor/ml. enzyme soln.	Hemoglobin	Units trypsin/ml. enzyme soln.		Inhibition
			$\times 10^{-4}$ without inhibitor	$\times 10^{-4}$ with inhibitor	
0.10	0.05	<i>per cent</i>			<i>per cent</i>
		2.2	43.5	14.5	66.7
		1.76	44.6	14.5	67.5
		1.32	43.8	15.1	65.5
		1.1	48.9	14.9	69.5

was not affected by variations of the concentration of hemoglobin within the range of 1.1–2.2% hemoglobin. These results indicate that the inhibitor does not act by combining directly with the substrate nor does it compete with trypsin for the substrate.

TABLE II  
*Inhibition of Various Amounts of Trypsin with a Constant  
Amount of Inhibitor and Substrate*

Ml. stock trypsin/ml. enzyme soln.	Units trypsin/ml. enzyme soln.		Inhibition
	no inhibitor	$\times 10^{-4}$ 0.05 ml. inhibi- tor/ml. enzyme soln.	
0.25	51.2	26.9	<i>per cent</i> 47.5
0.20	39.4	17.3	56.1
0.15	29.6	11.5	61.2
0.10	22.1	7.2	67.4
		0.025 ml. inhibitor/ml. enzyme soln.	
0.10	44.1	22.6	48.8
0.075	37.8	17.5	53.7
0.05	25.8	11.1	57.0
0.025	14.5	5.1	64.8
0.05	21.5	10.4	51.6
0.04	19.1	7.1	62.8
0.03	15.9	5.5	65.4
0.02	11.0	3.7	66.3
0.01	8.0	2.3	71.2

Table II presents the results when substrate and inhibitor were constant and trypsin varied. These data indicate that, as the trypsin concentration was decreased, the extent of inhibition was increased. The inhibitor, hence, must act upon the trypsin either to prevent its combination with the substrate or to prevent the dissociation of the enzyme-substrate complex.

Applying these results to an experiment with constant amounts of trypsin and substrate, increasing amounts of inhibitor would be expected to result in increased inhibition. Now if the inhibitor acts upon the trypsin to remove it stoichiometrically, successive increments of

inhibitor would increase the inhibition by successive constant amounts until inhibitor exceeds trypsin. If such results were plotted, a straight line would result. But if the inhibitor acts upon the trypsin to form an equilibrium complex, successive increments of inhibitor would have decreasing effects. If such results were plotted, an asymptotic curve would result. The results from such an experiment with constant amounts of trypsin and substrate and various amounts of inhibitor are recorded in Table III. The data indicate that the reaction involving trypsin and inhibitor is an equilibrium reaction.

TABLE III  
*Inhibition of Constant Amounts of Trypsin and Substrate by  
Various Amounts of Inhibitor*

Ml. stock trypsin/ ml. enzyme soln.	Ml. inhibitor/ml. enzyme soln.	Units trypsin/ml. enzyme soln.	Inhibition	Units inhibitor/ml. inhibitor soln.
		$\times 10^{-3}$	per cent	$\times 10^{-3}$
0.15	0.0	72.7	00.0	
	0.025	57.3	21.2	
	0.05	46.3	36.3	
	0.075	39.5	45.6	
	0.1	40.3	44.5	2.3
0.10	0.0	47.1	00.0	
	0.025	34.6	26.5	
	0.05	24.5	48.0	
	0.075	22.3	52.7	
	0.1	22.7	51.8	2.1
0.05	0.0	26.3	00.0	
	0.025	13.4	49.1	
	0.05	12.4	52.9	
	0.075	12.6	52.1	
	0.1	12.0	54.4	2.4

#### *A Unit of Inhibitor*

When the values of Table III are plotted on semi-logarithmic paper, units of trypsin on the logarithmic axis and ml. of inhibitor on the plain axis, the resulting curve approaches a straight line for small quantities of inhibitor. From such a curve the point of 25% inhibition can readily be found. This point is proposed as the index point of the

inhibitor unit. One unit of inhibitor is defined as that amount which will reduce the action of the trypsin from one trypsin unit (9) to 0.75 trypsin unit. That is, if  $72.7 \times 10^{-6}$  trypsin units are inhibited 25% or reduced to  $54.5 \times 10^{-6}$  units by 0.032 ml. of inhibitor solution, then there are  $72.7 \times 10^{-6}$  inhibitor units in 0.032 ml. inhibitor solution or  $2.3 \times 10^{-3}$  inhibitor units/ml. of inhibitor solution.

#### *Effect of Incubating Trypsin and Inhibitor*

To investigate the effect of trypsin on the inhibitor, or *vice versa*, over a period of time, 1 ml. of stock trypsin was mixed with 0.5 ml. of inhibitor solution, adjusted to pH 7.5, and diluted to 10 ml. This was then incubated at 37°C. and trypsin determinations made at intervals. The results appearing in Table IV indicate that the inhibition of trypsin

TABLE IV

*Effect of Incubating Trypsin with the Inhibitor before Making Trypsin Assay*

Incubation hours	Units inhibitor/ml. inhibitor soln. $\times 10^{-3}$
0	1.5
1	1.9
6	1.4
24	1.6

was not affected by incubating trypsin and inhibitor prior to adding to the substrate.

#### *Method of Extracting Inhibitor from Soybean Meal*

In Table V are recorded the results of extracting unheated, solvent-extracted soybean meal with various concentrations of hydrochloric acid. One part of meal was mixed with 10 parts of the extractant, placed in the ice box overnight, and insoluble materials removed by centrifugation. Inhibitor, pH and nitrogen were determined on the decantate. As was expected, the least amount of nitrogen was extracted at about pH 4.2, the isoelectric point for most of the soybean proteins (11). However, the pH had little effect on the amount of inhibitor extracted. When the soybean meal was extracted for different periods of time with 0.05 *N* hydrochloric acid, it was found that 2

hours was sufficient time to extract the inhibitor. It should be noted that, although one extraction removes a large fraction of the inhibitor from the soybean meal, successive extractions are required to remove all of the inhibitor.

TABLE V  
*Effect of Acidity on Extraction of Inhibitor and Nitrogen*

Hydrochloric acid	pH of extract	Mg. N <sub>2</sub> /ml. extract	Inhibitor units/ml. of extract
<i>N</i>			$\times 10^{-3}$
0.5	0.9	2.96	3.1
0.1	2.9	3.44	3.7
0.075	3.6	1.03	
0.050	4.25	0.83	3.8
0.025	4.9	1.00	
0.01	5.6	1.85	3.7
0.005	5.8	2.73	
0.001	6.1	3.42	3.5
0.0001	6.2	3.68	
0.00001	6.1	3.65	
0.000001	6.2	3.66	
Water	6.2	3.73	3.3

*Heat Destruction of the Inhibitor and of Urease*

To study the heat destruction of the inhibitor and of urease, samples of unheated solvent-extracted soybean meal were heated in the autoclave or electric oven in a metal pan in a layer approximately 3 mm. deep. Tests were made for the inhibitor as outlined previously and for urease as directed by Caskey and Knapp (3). The results expressed as *per cent* of the inhibitor remaining active and tests for the presence or absence of urease are recorded in Table VI. As much as 45% of the inhibitor remained active even though a negative urease test was obtained. Hence, the urease test must be regarded as unsatisfactory for testing for adequacy of heat treatment of soybean meal. Twenty-eight *per cent* of the inhibitor was still active in a sample of soybean meal heated in the oven at 135°C. for four hours. This showed the relative ineffectiveness of dry heat in destroying the inhibitor although the meal was distinctly "toasted" by such heating.

TABLE VI  
Heat Destruction of Inhibitor<sup>1</sup> and Urease<sup>2</sup>

Time	Flowing steam	Autoclave lb./sq. in.				Oven 135°C.
		5	10	15	20	
<i>min.</i>						
5				81+	73+	
7					9-	
10			59+	43-	0-	
15		87+	45-	20-		
20			21-	0-		
30	62+	41-	0-			
45	38-	19-				
60	22-	0-				78+
90	0-					
120						57+
240						28-

<sup>1</sup> Inhibitor values expressed in *per cent* remaining active.

<sup>2</sup> Urease values expressed as present or positive test "+" and as absent or negative test "-."

### SUMMARY

1. The inhibitor of soybeans was found not to compete with trypsin for the substrate. The reaction involving trypsin and inhibitor was shown to be an equilibrium reaction.

2. A unit of inhibitor was defined as that amount which will reduce the action of trypsin from 1 trypsin unit to 0.75 trypsin unit.

3. The inhibitor was not affected in its action by incubating with trypsin for as long as 24 hours before assay.

4. The inhibitor was extracted from soybean meal by dilute hydrochloric acid in a short time. The least amount of protein was extracted at pH 4.2.

5. The urease test was not suitable for determining the adequacy of heat treatment of soybeans as measured by the inhibitor test. Conditions for complete destruction of the inhibitor by autoclaving were found. Dry heat was not as effective as moist heat in destroying the inhibitor.

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# The Biological Availability of l-Ascorbyl Palmitate\*

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Fatty acid monoesters of *l*-ascorbic acid have been prepared and described by Swern, Stirton, Turer, and Wells (1). These authors pointed out that, since these esters are probably esters of the primary hydroxyl group only, the structures concerned with antioxidant and antiscorbutic properties have presumably remained unaltered. On the presumption that these properties are intact, the same authors called attention to the possible uses of the ascorbyl esters in the food industry as antioxidants in dry fats and oils, as interfacial modifiers by virtue of the presence of both hydrophilic and lipophilic groups, and as a source of ascorbic acid.

The antioxidant properties of the monoesters of *l*-ascorbic acid for fats and oils have been reported by Riemenschneider, Turer, Wells, and Ault (2). Before one of these esters, for example, *l*-ascorbyl palmitate, can be used by the food industries the absence of any public health hazard must be established with a reasonable degree of certainty, and its value as a source of vitamin C should be shown. The recent report of Fitzhugh and Nelson (3) demonstrated the safety of using *l*-ascorbyl palmitate in foods. These authors showed that rats fed diets containing 2% *l*-ascorbyl palmitate for 9 months, and 0.25% for 2 years showed no evidence of toxicity as judged by growth rates and histological appearance of various tissues. The present paper reports data on the utilization of *l*-ascorbyl palmitate and equivalent amounts of pure ascorbic acid by scorbutic guinea pigs.

It has been concluded by Robison (4) and confirmed by Gould and Shwachman (5) that the serum phosphatase activity in guinea pigs is

\* The *l*-ascorbyl palmitate was supplied by the Eastern Regional Research Laboratory.



of osteoblastic origin, and that in scurvy there is an impairment of osteoblastic activity with a concomitant rise or fall in the blood serum phosphatase depending on whether or not ascorbic acid supplements are administered in conjunction with the scorbutogenic diet. Gould and Shwachman have shown that ascorbic acid in doses of 0.25 to 12 mg. administered daily for 5 days to scorbutic animals with lowered serum phosphatase levels resulted in a prompt increase of serum phosphatase. We have applied this procedure in evaluating the biological availability of equivalent amounts of *l*-ascorbyl palmitate and ascorbic acid.

Male guinea pigs weighing under 300 g. were placed on a scorbutogenic diet consisting of equal parts of dry skim milk (heated at 100°C. for 12 hours), rolled oats, and bran. The diet was fed *ad libitum* and was supplemented every 4 days with 1 cc. of U.S.P. cod-liver oil. The animals were maintained on this diet for about 25 days, or until the serum phosphatase values had fallen to low levels ranging from 3.2 to 8.8 units/100 cc. of serum.

Under light ether anesthesia, blood was withdrawn into Wassermann tubes, by direct cardiac puncture, allowed to clot, and centrifuged. For the determination of serum phosphatase activity the methods of Shinowara, Jones, and Reinhart (6), and Gould and Shwachman (5) were used with the following modification.

For total "alkaline" serum phosphatase 0.1 cc. of serum was added to 1 cc. of Gould and Shwachman substrate and incubated for 1 hour at 38°C. For inorganic serum phosphate 0.2 cc. of serum was used. To each tube, enough 10% trichloroacetic acid was added to bring the final volume to 2 cc. The tubes were centrifuged, and 1 cc. each of the clear supernatant liquids was transferred to a 25 cc. Erlenmeyer flask. Five cc. of 0.05 *N* sodium hydroxide was added to the sample for total serum phosphatase, and 5 cc. of 0.1 *N* sodium hydroxide to the sample for inorganic serum phosphate. To both flasks and to a third containing 6 cc. of distilled water were added 2-cc. portions of diluted molybdic acid reagent (one volume of 7.5% sodium molybdate plus one volume of 10% sulfuric acid), followed by 2-cc. portions of freshly prepared dilute stannous chloride solution (0.2 cc. of a 60% solution of  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  in conc.  $\text{HCl}$ , diluted to 100 cc. with distilled water). The flasks were agitated briefly and allowed to stand 6 minutes for color development. Readings were then made in a Klett-Summerson photoelectric

colorimeter using a color filter having transmission limits of 640-670 m $\mu$ .

Twelve scorbutic guinea pigs, with phosphatase levels ranging from 3.2 to 8.8 units/100 cc. of serum, were divided as follows: Six animals were given 0.5 mg. of ascorbic acid in 0.25 cc. water. Five animals received 1.18 mg. of *l*-ascorbyl palmitate ( $\approx$ 0.5 mg. ascorbic acid) dissolved in 0.25 cc. of propylene glycol, and one animal received 0.25 cc. of propylene glycol only. Four control guinea pigs on a non-scorbutogenic diet were given 1.18 mg. of *l*-ascorbyl palmitate in 0.25 cc. propylene glycol. All the above supplements were administered by mouth daily for 5 days. On the sixth day the serum phosphatase values were redetermined.

All data on serum phosphatase values on control animals and on scorbutic animals before and after administration of equivalent amounts of pure ascorbic acid or *l*-ascorbyl palmitate are summarized in Table I.

While the phosphatase values of the various animals extend over a rather wide range both before and after receiving supplements, the important fact is that each animal served as its own control. Two of the non-scorbutic controls showed increased phosphatase values and two showed decreased values after the administration of *l*-ascorbyl palmitate. The difference in phosphatase values of the one scorbutic control animal receiving propylene glycol is less than any of the differences in the non-scorbutic control group. In every instance a scorbutic animal receiving either ascorbic acid or *l*-ascorbyl palmitate responded with an increased phosphatase value, and the range of the increases is comparable in the two groups. While a rigid proof of the biological equivalence of equimolecular amounts of ascorbic acid and *l*-ascorbyl palmitate would require the determination of the minimum critical dose of each compound, the present data strongly suggest that the curative properties of *l*-ascorbyl palmitate in scorbutic guinea pigs is about equal to that of ascorbic acid. The alleviation of the gross symptoms of scurvy was comparable in the two groups of animals.

Since *l*-ascorbyl palmitate is soluble in fats and fat solvents, the demonstration that its biological utilization is comparable to that of ascorbic acid points to its use under conditions where the water soluble ascorbic acid would be less feasible. For example, *l*-ascorbyl palmitate added to cod-liver oil might serve the double purpose of supplying

TABLE I

*Phosphatase Activity\* of Blood of Normal and Scorbutic Guinea Pigs, Treated with Ascorbic Acid or l-Ascorbyl Palmitate*

Pig No.	Diet	Units of Phosphatase		Supplement Fed
		Before	After	
14	S	5.6	3.8	0.25 cc. propylene glycol daily.
2	S	6.5	8.6	0.5 mg. ascorbic acid in 0.25 cc. glass distilled water daily for 5 days.
3	S	7.3	8.3	
17	S	3.3	21.9	
19	S	4.4	11.8	
24	S	3.2	9.9	
15	S	6.9	11.8	
Mean		5.3	12.1	
11	S	8.8	15.8	1.18 mg. l-ascorbyl palmitate ( $\approx$ 0.5 mg. ascorbic acid) in 0.25 cc. propylene glycol daily for 5 days.
12	S	8.8	16.1	
18	S	4.0	6.6	
21	S	3.8	15.2	
23	S	4.1	17.1	
Mean		5.9	14.2	
3L	C	10.6	16.4	
R	C	12.1	9.5	
2R	C	20.0	15.4	
3R	C	12.5	13.6	
Mean		13.8	13.7	

\* Phosphatase unit as defined by Gould and Shwachman (5).

S. Scorbutic diet: equal parts of dry and baked skim milk powder, rolled oats, and bran.

C. Control diet: Purina Rabbit Chow, supplemented with fresh cabbage.

vitamin C and protecting vitamin A through its antioxidant properties. It has been shown by Bickoff, Williams, and Sparks (7) that l-ascorbyl palmitate caused a 3-fold increase in stability of carotene in lard, cottonseed oil, and coconut oil.

## SUMMARY AND CONCLUSION

A comparison of the biological availability of ascorbic acid and *l*-ascorbyl palmitate has been made, based upon the increase in serum "alkaline" phosphatase of scorbutic pigs.

*l*-Ascorbyl palmitate was found to have antiscorbutic activity comparable to an equivalent amount of ascorbic acid, 2.36 mg. of the ester being equivalent to 1 mg. of ascorbic acid.

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## Effect of Dihydroxystearic Acid on Vitamin K Synthesis by Rats \*

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### INTRODUCTION

It has been established (1) that there is no change in the prothrombin content of the blood of rats fed experimental diets devoid of detectable amounts of vitamin K. This appears to be due to a synthesis of vitamin K by the bacteria present in the intestinal tract. The amount of the vitamin produced by the intestinal flora varies markedly with the types of bacteria present, and this, in turn, can be modified by relatively small changes in the diet (2). While the coliform bacteria consistently found in the intestinal tract can synthesize this fat-soluble anti-hemorrhagic factor, many other bacterial types have this property (3). Synthesis takes place wherever these bacteria are growing, and the site of greatest synthesis in the rat has been reported to be in the caecum (2).

Intestinal synthesis of vitamin K is affected by bacteriostatic or bactericidal agents given orally to the rat. Sulfonamides added to experimental diets have produced a deficiency which could be prevented by feeding vitamin K (3, 4, 5). Although the total bacterial count was not changed, the number of *E. coli* was temporarily reduced and the enterococci increased (6).

We have previously reported (7) that vitamin K deficiency resulted when a diet containing dihydroxystearic acid was fed to rats. Since the deficiency was prevented and cured by the administration of small amounts of 2-methyl-1,4-naphthoquinone, it is likely that the syndrome did not result from an impairment of the absorption of vitamin

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K. This synthetic vitamin K is much less soluble in water and much more soluble in fat than the compound synthesized in the intestinal tract (8). Thus, the prevention or cure of the syndrome by synthetic vitamin K (2-methyl-1,4-naphthoquinone) is not final proof that the dihydroxystearic acid had no effect on the absorption of the vitamin K produced in the intestinal bacteria. We are reporting here the results of studies made to determine whether the vitamin K deficiency resulting from the feeding of dihydroxystearic acid is due to a bactericidal or bacteriostatic action of the hydroxy acid upon intestinal bacteria, or to a blocking of the biochemical process by which these bacteria synthesize vitamin K.

### EXPERIMENTAL

Four series of experiments were carried out on Sprague-Dawley weanling albino rats. In each series the 27 animals were divided into 3 groups so that the starting weights of each group averaged the same (usually 50 g.). In two of the series females were used, while in the other two only males were used. Each animal was caged individually over wide-mesh screening in order to minimize coprophagy. Distilled water was supplied *ad libitum*. In each series one group was fed a synthetic diet containing "reconstituted" hydrogenated vegetable oil, and the other two groups were given a similar diet containing dihydroxystearic acid. One of these latter groups was given a vitamin K supplement in addition (Table I).

The control or "reconstituted" fat was prepared by saponifying a quantity of hydrogenated vegetable oil, then re-esterfying it at 220°C. in the presence of carbon dioxide and 0.2% sodium hydroxide (based on weight of total fatty acids). The triglyceride containing dihydroxystearic acid was prepared by adding sodium dihydroxystearate to a saponified batch of the same hydrogenated vegetable oil, then esterifying in the same manner as the "reconstituted" fat. The quantity of sodium dihydroxystearate added was such that one-third of the fatty acid content of the resulting triglyceride was dihydroxystearic acid. The test diet contained 8% of dihydroxystearic acid since the triglyceride contained 32% of this fatty acid.

Samples of whole blood were taken from the tail at weekly intervals and the clotting time determined by a capillary method (10). The clotting time of normal rats ranged between 1.8 and 2.2 minutes. A clotting time longer than 10 minutes was interpreted to indicate a considerable decrease in the prothrombin level of the blood. When the clotting times of the rats receiving the diet containing dihydroxystearic acid were prolonged and death from loss of blood was imminent, all three groups of rats in each series were killed by decapitation.

The caecum and colon of each rat were aseptically stripped so that bacteriological and vitamin K analyses could be made on the contents. By an aseptic technique, the contents of the caecum were mixed with an equal weight of physiological saline (0.9% NaCl), and the contents of the colon were macerated with two parts by weight of saline. Bacteriological dilutions ranging between 1/100 and 1/100,000,000 were made, and one cc. of each dilution was surfaced on eosin-methylene blue agar plates

and tomato agar plates. The former plates were then incubated for 48 hours at 37°C. and the latter for 72 hours at 37°C. under 10% carbon dioxide. Difco dehydrated tomato juice agar was used after supplementation with 4 g. of agar and 5 g. of yeast extract. These additions seemed necessary because of the softness and instability of the medium due to its high acidity.

The vitamin K in the caecum and colon contents was estimated by the colorimetric oxidation and reduction method of Scudi and Buhs (11, 12).

TABLE I  
*Composition of Experimental Diets*

Diet component	Control diet	Dihydroxystearic acid diets	
	<i>g./100 g.</i>	<i>g./100 g.</i>	<i>g./100 g.</i>
Casein (Labco)	18	18	18
Sucrose	53	53	53
Salt Mixture (10)	4	4	4
Reconstituted hydrogenated vegetable oils	25	0	0
Dihydroxystearic acid triglyceride	0	25	25
Vitamin Supplement	<i>mg./day</i>	<i>mg./day</i>	<i>mg./day</i>
2-Methyl-1,4-naphthoquinone	0.0	0.0	0.1
Thiamine	0.02	0.02	0.02
Riboflavin	0.025	0.025	0.025
<i>d</i> -Calcium pantothenate	0.10	0.10	0.10
Pyridoxine	0.02	0.02	0.02
Niacinamide	0.10	0.10	0.10
Choline	15.0	15.0	15.0
Vitamin A	60 I.U.	60 I.U.	60 I.U.
Vitamin D	6 I.U.	6 I.U.	6 I.U.

Note: Vitamins A and D were dissolved in mineral oil and the other vitamins dissolved in water. Both preparations were delivered directly into the mouth by calibrated medicine droppers.

## DISCUSSION OF RESULTS

During the first part of each experimental period a severe diarrhea was noted in 100% of the male rats and only 20% of the female rats in the groups fed dihydroxystearic acid in the diet. In the groups receiving an addition of 2-methyl-1,4-naphthoquinone in the diet, a much milder form of diarrhea occurred in 50% of the males and 15% of the females. In all experiments the diarrhea disappeared within 14 days. Since this diarrhea occurred only in the groups fed dihydroxystearic acid, but was milder in groups receiving vitamin K supplements, it appears that the diarrhea is a manifestation of dihydroxy-



stearic acid toxicity, and that this toxicity is increased with concurrent avitaminosis K. It is interesting that castor oil, which is known to have a purgative effect, contains 1.8% dihydroxystearic acid (13), and that butterfat contains 1% of hydroxy-acid (14).

Reduced weight gain is apparently not a feature of vitamin K deficiency (15). The food consumption and weight increase of all groups were small (Table II) presumably due to the synthetic nature of the

TABLE II  
*Food Intake and Weight Increase*

Diet	Food consumption		Weight increase	
	males	females	males	females
Control	60	54	11	10
Dihydroxystearic acid without vitamin K	45	53	8	7
Dihydroxystearic acid with vitamin K	53	52	9	8

diets. While the addition of vitamin K to the diet of groups fed the hydroxy fat prevented the development of vitamin K deficiency, it did not alter the growth rate. Thus, the poorer growth performance of rats fed the triglyceride containing dihydroxystearic acid is evidence of toxicity.

Vitamin K deficiency and hypoprothrombinemia developed more rapidly in the male than in the female rats fed the dihydroxystearic acid diet (Table III). While the males required 30-42 days, the females reached the same state of avitaminosis only after 56-63 days. It is hardly likely that the diarrhea was responsible for this difference, for the development of the deficiency syndrome had no direct relation to the severity of the diarrhea in rats of the same sex in the same group.

The animals which had been fed the diets containing hydroxy fatty acid showed retarded sexual development in comparison with the control groups. The vaginas of most females were not opened at the end of the experiment, and the testes of the males were underdeveloped. Extensive internal hemorrhages were evident on autopsy. Griffith and Farris (16) reported that 60% of the vaginas of 262

TABLE III  
Blood Clotting Times

Sex	Experimental period	Average blood clotting time		
		Control diet	Dihydroxystearic acid diets	
			without vitamin K	with vitamin K
	<i>days</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
Male	30	1.8	15.0	2.2
Male	42	2.0	20.0	2.0
Female	56	2.2	22.0	2.0
Female	63	2.2	23.0	1.8

female rats were open at 35 to 50 days, and 85% of the testes of 250 male rats were descended between 18 and 31 days of age. In the present investigation the males were 65-77 days old and the females were 91-98 days old when the experiment was ended. Apparently the dihydroxystearic acid is specifically responsible, for the same retardation in sexual development was noted when vitamin K was fed in the dihydroxystearic acid diet.

The inclusion in the diet of the triglyceride containing dihydroxystearic acid had no effect upon the kinds of bacteria present in the intestines (caecum and colon) of the rats in each series. Furthermore, the total number of each bacterial type was not affected by the inclusion of the hydroxy fat in the diet. The dihydroxystearic acid did not alter the kinds or quantities of intestinal bacteria, and thus did not exert a bacteriostatic or bactericidal effect upon the intestinal flora.

The contents of the caecum of the rats fed the control fat were found to contain 19-23  $\gamma$  of vitamin K/g. (wet weight basis), while the contents of the colon contained 11-14  $\gamma$ /g. The caecum and colon of rats fed the hydroxy fat contained no detectable vitamin K (Table IV). The absence of measurable vitamin K in the intestines of these rats indicates that the dihydroxystearic acid stopped the synthesis of vitamin K by intestinal microorganisms. This demonstrates that intestinal bacteria do synthesize vitamin K *in vivo* and that dihydroxystearic acid blocks the mechanism involved in the synthesis of vitamin K.

This research demonstrates also that the rat has a physiological requirement for vitamin K. On normal diets this requirement may be

TABLE IV

*Average Concentration of Vitamin K in the Contents of the Caecum and Colon*

Diet	Male		Female	
	contents of			
	caecum	colon	caecum	colon
Control Dihydroxystearic acid without vitamin K	23 negative	12 negative	19 negative	13 negative

met by intestinal synthesis. It is likely that the vitamin K requirement of the male rat exceeds that of the female rat.

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## SUMMARY

1. A triglyceride containing dihydroxystearic acid produced a vitamin K deficiency within 30-60 days when fed to weanling rats. This deficiency was prevented by feeding 0.1 mg. of 2-methyl-1,4-naphthoquinone daily.

2. The deficiency developed more rapidly in male than in female rats.

3. No measurable vitamin K was present in the intestinal tracts of rats fed a triglyceride containing dihydroxystearic acid, indicating that the intestinal synthesis of vitamin K was arrested.

4. The deficiency syndrome was not the result of a bactericidal or bacteriostatic action by the dihydroxystearic acid, for the kinds and numbers of intestinal bacteria were not affected.

5. Dihydroxystearic acid affects the vitamin K metabolism of bacteria, presumably by blocking the biochemical system involved in the synthesis of vitamin K.

6. Rats have a physiological requirement for vitamin K which normally is supplied by intestinal synthesis. The requirement of male rats is apparently higher than that of female rats.

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# Availability of Vitamins in Foods and Food Products

## III. Riboflavin Balances with Liquid Milk, Dried Skimmed Milk, and Evaporated Milk and the Influence of Succinyl Sulfathiazole on Such Balances <sup>1</sup>

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### INTRODUCTION

As cow's milk is one of the richest sources of riboflavin in our daily food supply, a study was carried out on the efficiency of utilization of this vitamin in liquid cow's milk, dried skimmed milk, and evaporated milk. The cow's milk was obtained from the University dairy, the dried skimmed milk from the Borden Company, New York and the evaporated milk from a local grocery store. To reduce bacterial synthesis, experiments were also conducted on the influence of the addition of 2% succinyl sulfathiazole in the purified ration on fecal riboflavin excretions.

### EXPERIMENTAL

The method of riboflavin determination used was that of Conner and Straub for the feces (1) and modifications of the method of Hodson and Norris (2) for the riboflavin content of urine. The riboflavin content of the liquid and evaporated milk was determined bi-daily. The research was conducted on albino rats.

The animals were fed a purified diet of the following percentage composition: vitamin-free casein (Smaco), 18; roughage (Cellu-flour), 2; Salts No. 1 (3), 4; butter fat, 10; and dextrose, 66. The vitamin supplements were administered daily separately from the ration as follows: 20  $\gamma$  thiamine, 20  $\gamma$  pyridoxine, 100  $\gamma$  calcium pantothenate, and 6 mg. choline chloride. As a source of vitamin A and D, three drops of halibut liver oil were given once weekly to each animal.

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<sup>1</sup> Research paper No. 822, Journal Series, University of Arkansas. Published with the permission of the Arkansas Agricultural Experiment Station. Aided by a grant from the Committee of Scientific Research of the American Medical Association.

The riboflavin balance studies in urine and feces were carried out on three groups of animals, 24 in each group consisting of an equal number of male and female rats. The animals were started on experiments when about six weeks of age, weighing 80 to 100 g. each. The animals were depleted for five to six weeks until the urinary riboflavin weekly excretions reached a constant minimum of 15–16  $\gamma$  and the weekly fecal excretion of this vitamin about 40  $\gamma$ .

The results of this study are presented in summarized form in Table I.

*Riboflavin Balances with Liquid Cow's Milk, Dried Skimmed Milk and Evaporated Milk*

Riboflavin balances in urine and feces were carried out for 14 days in 12 animals which received daily about 13 cc. of cow's milk and 12 other rats which were given daily pure crystalline riboflavin equal to that found in the milk. The same groups of animals were then continued for another 14 days metabolism studies during which period they received 2% succinyl sulfathiazole in the ration. Similar procedures with equal numbers of animals were used in group 2 for the dried skimmed milk, and in group 3 for the evaporated milk (Pet). However, in the latter group the sulfonamide drug was not introduced. In order to circumvent complications of the influence of the plane of nutrition on the riboflavin balances, all animals were fed daily 8 g. of the purified ration.

It will be noted that the animals which received liquid cow's milk, dried skimmed, or evaporated milk, as sources of riboflavin, excreted considerably more riboflavin in urine and feces than the rats which received equivalent amounts of pure crystalline riboflavin daily throughout the experimental periods. That the large fecal riboflavin excretions when milk was fed as a source of the vitamin are due largely to bacterial synthesis is evident from the reduction following the introduction of 2% succinyl sulfathiazole in the purified ration (Table I). The sulfa drug produced some increase in urinary excretions of riboflavin but it was relatively small and is probably not significant. However, the question arises, what is the significance of the large urinary excretions of riboflavin when cow's milk served as the only source of this vitamin? The fact that such animals gained about twice as much weight during the metabolism periods as the rats which had equivalent amounts of the pure vitamin, also since the riboflavin in milk exists

TABLE I  
*Riboflavin Balances with Milk and the Influence of  
 Succinyl Sulfathiazole on Such Balances<sup>1</sup>*

Milk or pure riboflavin	Group number	Number of animals	Metabolism period	Total riboflavin intake	Change in body weight	Riboflavin excretions			
						In urine <sup>2</sup>		In feces <sup>2</sup>	
			days	g	g	g	Per cent of T.I. <sup>3</sup>	g	Per cent of T.I. <sup>3</sup>
Cow's milk	1-A	12	14	321	+14.9	153	47.7	66	20.6
Pure riboflavin	1-A	12	14	321	+6.7	57	17.7	20	6.2
Cow's milk +2% sulfonamide in ration	1-B	12	14	341	+13.7	181	53.1	39	11.5
Pure riboflavin +2% sulfonamide in ration	1-B	12	14	341	+7.8	63	18.5	0	0.0
Dried skimmed milk	2-A	12	14	280	+10.4	124	44.3	77	27.3
Pure riboflavin	2-A	12	14	280	+6.7	45	16.1	24	8.6
Dried skimmed milk +2% sulfonamide in ration	2-B	12	14	280	+8.8	144	51.1	30	10.7
Pure riboflavin +2% sulfonamide in ration	2-B	12	14	280	+5.8	66	23.5	6	2.1
Evaporated milk	3	24	14	329	+16.1	87	26.4	91	27.6
Pure riboflavin	3	24	14	329	+7.1	10	3.0	10	3.0

<sup>1</sup> Figures in this table represent averages per animal.

<sup>2</sup> Corrected for the amount excreted on a riboflavin deficient ration.

<sup>3</sup> T. I. = Total intake.

almost entirely in the free state, would indicate that such large excretions are not due to poor absorption. In order, however, to be absolutely certain about this point, another investigation was carried out with weanling rats transferred on experiments from another type of diet during lactation.

It was anticipated that the large urinary riboflavin excretions from milk may be due to elimination of excess needs rather than due to poor retention. Our cereal stock diet is supplemented with an abundance of cow's milk daily. Hence, there is considerable storage of riboflavin from such diet during the nursing period and it takes several months before weanling rats from this ration will reach maintenance. Therefore, two



litters of weanlings were transferred from a diet containing 74% polished rice and 15% peanut meal. As a source of riboflavin, the maternal diet during lactation was supplemented with 100  $\gamma$  of this pure vitamin daily. Such a dietary regime produced little storage of riboflavin in nursing young. The average gain per animal during the five weeks depletion periods was less than 6.7 g. or 1.26 g./week/rat. After the riboflavin depletion period, four animals were given daily 250 mg. dried skimmed milk, equivalent to 5  $\gamma$  riboflavin, and another four animals were given 5  $\gamma$  of the pure vitamin. After four weeks the animals which received the dried skimmed milk as a source of riboflavin made a total gain of 104 g. and ate 508 g. of food. The rats which received the equivalent amount of pure vitamin gained 103 g. and ate 544 g. of the same ration. From these results it is evident that the riboflavin in dried skimmed milk is as efficiently utilized as the equivalent amount of the pure crystalline vitamin.

Since during this war it became increasingly difficult to purchase commercial casein, which is a constituent of our cereal stock ration, for several months we incorporated 18% dried buttermilk in such ration as a protein supplement. When, however, we attempted to deplete rats weaned from mothers which had our stock diet containing the dried buttermilk and which ration was also supplemented daily with cow's milk, 18 rats were still growing after 5 months depletion of riboflavin with no external sign of such avitaminosis. One animal died after 119 days depletion. After 124 days depletion one animal lost 44 g. after having gained 68 g. from the initiation of the experiment. Following administration of 250 mg. dried skimmed milk, which provided 5  $\gamma$  riboflavin, there was a gain of 62 g. in three weeks.

#### SUMMARY

Riboflavin balance studies were conducted on albino rats which received cow's milk, dried skimmed milk, and evaporated milk as a source of this vitamin. The milk sources provided 20-25  $\gamma$  riboflavin daily. A comparison was made between the urinary and fecal excretions of riboflavin from milk and from that of the pure crystalline vitamin. The animals on the cow's milk, dried skimmed milk and evaporated milk excreted much larger amounts of riboflavin than the animals given the same amounts of the pure vitamin.

The larger fecal riboflavin excretions were considerably reduced by the introduction of 2% succinyl sulfathiazole in the experimental

ration, which indicates that a great portion of such large excretions was due to bacterial synthesis.

The large urinary riboflavin excretions from milk sources are due to elimination of excess needs rather than to poor utilization. This became apparent when weanling rats were transferred from a diet on which there was relatively little storage. After five weeks depletion, the same gains of weight were produced in subsequent four weeks following administration of 250 mg. dried skimmed milk, which supplied only 5  $\gamma$  riboflavin daily compared with 5  $\gamma$  daily administration of the pure vitamin. The conclusion, therefore, is that riboflavin in cow's milk is well utilized.

When the maternal diet was abundant in cow's milk and dried buttermilk there was considerable storage of riboflavin in the weaned young from such a dietary regime. No signs of riboflavin deficiency were detected in 18 rats weaned from such a diet after five months depletion when growth was still continuing.

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# Partial Purification and Properties of Tomatin, an Antibiotic Agent from the Tomato Plant<sup>1</sup>

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## INTRODUCTION

Tomatin,<sup>2</sup> an antibiotic agent present in the cultivated tomato plant (*Lycopersicon esculentum*), is capable of inhibiting the soil-borne fungus *Fusarium oxysporum* f. *lycopersici* that causes tomato wilt, an economically serious disease of this important food crop (1). Moreover, it has been found that the antibiotic effectiveness of tomatin is not limited to this one organism; it inhibits to an even greater extent cultures of certain human pathogenic fungi and bacteria (2).

The results of these preliminary investigations point to two interesting possibilities: (A) that tomatin may be responsible, either wholly or in part, for the resistance offered by certain tomato varieties to *Fusarium* wilt, which means that the elucidation of the chemical nature of tomatin and the mechanism of its action in the tomato plant may afford a new approach to the study of plant disease resistance and may contribute to the rapid development of more highly disease-resistant plant varieties; and (B) that because of its marked inhibitory action upon several important human pathogens (particularly the fungi), tomatin may be of therapeutic value in the treatment of human infections caused by these organisms.

The academic and practical significance of the possibilities that have been mentioned have encouraged us to make concerted efforts to isolate tomatin, to determine its structure, its origin, action, and fate in the

<sup>1</sup> Presented in part before the Division of Biological Chemistry of the American Chemical Society, Atlantic City, New Jersey, April, 1946.

<sup>2</sup> This antibiotic agent was originally designated "lycopersicin" (1), but this term has been abandoned for reasons indicated recently (2).

tomato plant, and its toxicity to animals and humans, and to extend the investigation of its antibiotic spectrum to the largest practicable number of different pathogenic organisms. The major part of this program remains to be accomplished. Tomatin has not been isolated as a pure compound. In fact, it cannot be stated with certainty at this stage in the investigation whether the antibiotic activity of tomatin is due to a single compound or to two or more compounds of somewhat similar characteristics. It will be appreciated, therefore, that the results to be reported are based on observations made with partially purified, but still impure preparations of tomatin and that, accordingly, the interpretation of many of the results must be regarded as tentative.

It is the purpose of this paper to present procedures for obtaining a crude and a partially purified tomatin preparation from tomato plants, to discuss some of the chemical, physical and toxicological characteristics of tomatin preparations, and to demonstrate the effect of tomatin upon cultures of a considerable number of microorganisms.

## EXPERIMENTAL

### *Procedure for Obtaining Tomatin Preparations*

Tomatin is present in highest concentration in tomato leaves, to a lesser extent in the roots, and to the least extent in the stems and fruit. In the laboratory preparation of tomatin it is preferable to use the leaves of the Red Currant tomato plant (*Lycopersicon pimpinellifolium*), a Peruvian variety that bears currant-sized fruit, since this variety is the richest source of tomatin so far discovered. For practical preparative purposes, however, whole plants of any commercial variety may be used as the source of tomatin. Depending upon its accessibility, the plant material may be processed either in the fresh condition or after drying and grinding. A typical experiment, illustrating the procedure used for processing dried, whole tomato plants for tomatin and the yields that are to be expected, is described below. In discussing the results of this experiment and others, tomatin activity is expressed in arbitrary units, which are estimated by means of the familiar cylinder-plate technique, *Fusarium oxysporum* f. *lycopersici* being used as the test organism and a sterile tomatin solution, prepared from tomato leaves and stored in sealed ampoules, being used as the reference standard (1). A solution containing one unit of tomatin/ml. will produce an inhibition zone of 18.5 mm. on the standard assay plate.

Two hundred g. of dried tomato plant, estimated to contain 4400 units of tomatin activity, were extracted with 4 successive 1-liter portions of hot methanol. The clear, green extract was concentrated to dryness *in vacuo* and the residue was thoroughly extracted, portionwise, with boiling water to yield approximately 500 ml. of clear, yellow solution. Upon evaporation to dryness *in vacuo* this extract yielded 21 g. of crude tomatin containing approximately 4000 units.

These results indicate that a tomatin preparation which is nearly 10 times as potent as the starting material can be obtained quite simply without much loss of activity.

When fresh plant material is to be processed, it is ground and pressed, and the juice is autoclaved at 120°C. for approximately one hour. The inactive precipitate which forms is separated, the clear extract is concentrated to dryness, and the residue is processed exactly as indicated above. In this manner tomatin preparations having the same potency as those obtained by processing dried material are obtained, but the yields are somewhat lower since the separation of the active juice from the fresh plant residue is inefficient unless excessive amounts of water are used.

Crude tomatin preparations contain approximately 0.2 unit of tomatin activity/mg. In numerous attempts to increase the potency of such preparations, fractions have been isolated which have potencies of 1 to 2 units/mg. Unfortunately, these procedures have resulted in such prohibitive losses in total activity that the procedures so far utilized have been temporarily abandoned as impractical.

It is obvious that potencies in the range of 1 to 2 units/mg. appear insignificant when tomatin is compared with certain other antibiotic agents. As will be apparent later, however, the arbitrary unit (based on *Fusarium* as the test organism) that is used for the evaluation of tomatin attributes deceptively low antibiotic activity to tomatin when it is compared with other antibiotic agents. This is due in large part to the fact that, of all the organisms so far tested, the *Fusarium* species are by far the least sensitive to the inhibitory action of tomatin. Nevertheless, we have adhered to the use of *Fusarium oxysporum* f. *lycopersici* as the test organism, because it is the activity of tomatin preparations toward various *Fusarium* species which will be of greatest significance in the study of wilt resistance in plants.

#### *Physical and Chemical Properties of Crude Tomatin Preparations*

In aqueous solution (pH 5.5) in sealed ampoules, crude tomatin is able to withstand a temperature of 120°C. for at least 5 hours without detectable loss of antibiotic activity. Tomatin is dialyzable. Based on the study of crude preparations, tomatin is soluble in water at pH values below neutrality, is very soluble in absolute methanol, and exhibits progressively decreased solubility in ethanol, isopropanol and butanol. Tomatin is insoluble in all other organic solvents tried including aromatic and aliphatic hydrocarbons, ketones, ethers and esters, chloroform and dioxane. The antibiotic agent is adsorbed from slightly acid aqueous solution on charcoal (Norit A) and on alumina. Tomatin can be eluted from charcoal by the use of acid aqueous methanol.

Under certain conditions tomatin is precipitated, or adsorbed on material precipitated from crude aqueous solutions when the solution is adjusted to pH values above neutrality. Although much inert material is eliminated in this manner, the loss of total tomatin activity by precipitation at alkaline pH values makes this method impractical for purification.

One preparation,<sup>3</sup> having a potency of 1.2 tomatin units/mg., has been analyzed. It is realized that the preparation analyzed was quite impure. However, no sulfur was detected and a nitrogen value of less than 1% was obtained.

The most highly active preparations of tomatin that have been obtained contain sufficient color in both water and alcohol solutions to prevent measurement of optical activity. Crude tomatin preparations exhibit a strongly positive orcinol test, indicating the presence of carbohydrate. Phenolic tests and the ninhydrin test are negative.

#### *Effect of Tomatin on Several Microorganisms<sup>4</sup>*

The effects of tomatin upon a considerable number of microorganisms are given in Tables I, II and III. It will be observed that the organisms

<sup>3</sup> Fresh tomato plant juice was autoclaved for 1 hour at 15 lbs. pressure. The insoluble precipitate which formed was removed by centrifugation and the supernatant liquid was then adjusted to pH 7.5 by adding a concentrated NaOH solution with continuous stirring. The precipitate was removed and the pH 7.5 mother-liquor was reserved for subsequent processing (see below). The precipitate was suspended in water and adjusted to pH 4.0 by adding HCl solution. Not all of the pH 7.5 precipitate dissolved at pH 4.0. The clarified solution (pH 4.0) was concentrated to dryness, extracted with absolute methanol, and the methanol extract was concentrated to dryness. The dry residue was extracted with a minimum amount of water and the water extract was lyophilized. The dried product (T-1) contained 1.2 tomatin units/mg.

The pH 7.5 mother-liquor, obtained as described above, was adjusted to pH 9.0 and the precipitate was recovered by centrifugation. The precipitate was suspended in water and adjusted to pH 4.0. Practically all of the precipitate dissolved. The clarified solution (pH 4.0) was then processed exactly as described for the preparation of T-1. The active material thus obtained (T-2) contained 1.2 units/mg. and was used in the toxicity tests and in part of the antibiotic spectrum studies reported in this paper.

<sup>4</sup> The following symbols have been used to identify the sources of the organisms used in these investigations (see Tables I, II and III).

ATCC—American Type Culture Collection.

HHF—H. H. Flor, Division of Cereal Investigations, Bureau of Plant Industry, Soils and Agricultural Engineering.

TABLE I

*Organisms Whose Growth is Completely Inhibited by the Presence of  
1 Unit Tomatin/ml. of Culture Medium*

Organism	Disease and remarks
<i>Candida (Monilia) albicans</i> , E3147 <sup>a b</sup>	Moniliasis (Thrush, Bronchomycosis)
<i>Cryptococcus neoformans</i> , E3708 <sup>a</sup>	Cryptococcosis (European Blastomycosis, Torulosis)
<i>Debaryomyces histolytica</i> , ATCC732 <sup>a</sup>	
<i>Trichophyton mentagrophytes</i> , ATCC9533 <sup>c</sup>	
<i>Trichophyton interdigitale</i> , E640	
<i>Trichophyton rubrum</i> , AMS	
<i>Trichophyton gypsum</i> , E666	The Dermatomycoses (Ringworm, Athlete's Foot, etc.)
<i>Epidermophyton floccosum</i> , E1207	
<i>Microsporum audouinii</i> , ATCC9082	
<i>Achorion gypsum</i> , ATCC6286	
<i>Achorion schoenleemii</i> , ATCC4822	
<i>Blastomyces dermatitidis</i> , E6014 <sup>a b</sup>	North American Blastomycosis
<i>Coccidioides immitis</i> , ATCC9180	Coccidioidomycosis
<i>Histoplasma capsulatum</i> , E6507	Histoplasmosis

<sup>a</sup> A tomatin concentrate assaying 0.2 unit activity/mg. solids was used for these tests. All other organisms were tested using tomatin preparation T-2.<sup>3</sup>

<sup>b</sup> Tested using both of the tomatin preparations described in (a) above.

have been divided into 3 groups depending upon whether they are completely inhibited, partially inhibited, or unaffected by the presence of 1 unit of tomatin/ml. in the culture medium. These results constitute only a rough screening test for the relative sensitivity of the organisms to tomatin and it is likely that in many cases complete or partial inhibition of growth would have occurred had far less tomatin been used. For instance, in the case of *Trichophyton mentagrophytes*, one of the organisms of the ringworm group affecting man, complete inhibition can be effected with as little as 0.2 unit tomatin/ml. of medium; in the case of *Blastomyces dermatitidis*, the organism associated with North American blastomycosis, complete inhibition is attained at a tomatin level of 0.05 unit/ml. Conversely, it is possible that inhibition of some of the organisms that appear to be resistant to the

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JCW—J. C. Walker, University of Wisconsin.

NRRL—Northern Regional Research Laboratory.

AMS—Army Medical School.

C—H. R. Curran, Bureau of Dairy Industry.

E—C. W. Emmons, National Institute of Health.

R-5-6—F. L. Wellman (3).



TABLE II  
Organisms Whose Growth is Partially Inhibited by the Presence  
of 1 Unit Tomatin/ml. of Culture Medium

Organisms	Disease and remarks
<i>Fusarium oxysporum</i> f. <i>lycopersici</i> , R-5-6 <sup>a</sup> <sup>b</sup>	Tomato wilt
<i>Fusarium oxysporum</i> f. <i>pisi</i> , JCW <sup>a</sup> <sup>b</sup>	Pea wilt
<i>Fusarium oxysporum</i> f. <i>conglutinans</i> , JCW <sup>a</sup> <sup>b</sup>	Cabbage yellows
<i>Fusarium oxysporum</i> f. <i>lini</i> , HHF343 <sup>a</sup> <sup>b</sup>	Flax wilt
<i>Actinomyces scabies</i> , ATCC3352	Potato scab
<i>Sporotrichum schenckii</i> , E7017	Sporotrichosis
<i>Monosporium apiospermum</i> , AMS	Maduromycosis (Maudra Foot, Mycetoma)
<i>Aspergillus niger</i> , ATCC6267	Rarely pathogenic
<i>Aspergillus clavatus</i> , ATCC1007	
<i>Penicillium notatum</i> , NRRL124B21	
<i>Staphylococcus aureus</i> , NRRLB313 <sup>a</sup>	
<i>Bacillus cereus</i> , C369 <sup>a</sup>	Gram-positive
<i>Bacillus mycoides</i> , C6462 <sup>a</sup>	Gram-positive
<i>Bacillus subtilis</i> , NRRL558 <sup>a</sup>	Gram-positive
<i>Escherichia coli</i> , NRRLB210 <sup>a</sup>	Gram-negative

<sup>a</sup> See footnote (a), Table I.

<sup>b</sup> See footnote (b), Table I.

TABLE III  
Organisms That are Unaffected by the Presence of 1 Unit  
Tomatin<sup>a</sup>/ml. of Culture Medium

Organism	Disease and remarks
<i>Actinomyces (Nocardia) asteroides</i> , ATCC3308	Actinomycosis (Lumpy Jaw)
<i>Actinomyces hominis</i> , ATCC3008	
<i>Phialophora verrucosa</i> , AMS	Chromoblastomycosis
<i>Hormodendrum pedrosoi</i> , AMS	

<sup>a</sup> Tomatin preparation T-2<sup>8</sup> used in all tests.

action of tomatin might be accomplished by the use of higher tomatin concentrations.

It is evident from these results that tomatin is highly effective against cultures of the human dermatophytic fungi and against several of the fungi and yeast-like forms that cause internal disease (4). It is slightly effective under the conditions employed against the tested species of bacteria, the fungi *Fusarium*, *Penicillium*, *Aspergillus*, *Sporotrichum*, and *Monosporium* and the plant pathogenic *Actinomyces*. It is without significant effect upon the human pathogenic *Actinomyces*, and the fungi responsible for the chromoblastomycoses.

An interesting difference exists between the results reported here for the inhibition of *Escherichia coli* and *Penicillium notatum* and those reported in the previous paper (2). When tomatin (1 unit/ml.) is present in the culture medium, *E. coli* and *P. notatum* are partially, but very definitely inhibited. However, neither organism is inhibited perceptibly by solutions containing as much as 5 units of tomatin/ml. in the cylinder-plate method. There is no obvious explanation for this behavior.

It should be emphasized that the results presented in Tables I, II and III are indicative of the inhibitory action of tomatin primarily

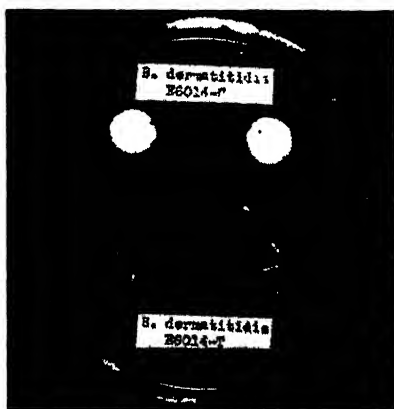


FIG. 1. Effect of Tomatin on *Blastomyces dermatitidis*. Upper half, control, no tomatin. Lower half, one unit of tomatin/ml. of medium.

upon the vegetative growth of the organisms tested. It is not feasible by the technique employed to observe accurately the fungicidal or bactericidal effects of tomatin. By the use of suitable methods, however, it has been possible to demonstrate that tomatin is definitely fungicidal for spores of *Fusarium oxysporum* f. *lycopersici*, even though it exerts only partial inhibition toward the vegetative proliferation of this same organism.

Typical test plates which illustrate the action of tomatin upon several of the organisms listed in Tables I and II are shown in Figs. 1 and 2.

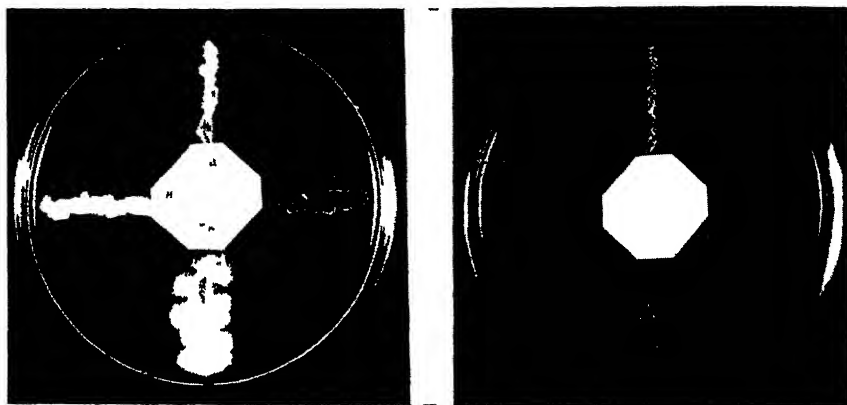


FIG. 2. Effect of Tomatin on *Staphylococcus aureus* (Sa), *Escherichia coli* (Ec), *Bacillus cereus* (Bc), and *Candida albicans* (Ca). Left, control (C), no tomatin. Right, one unit of tomatin (T1)/ml. of medium.

### Toxicity Tests

Preliminary toxicity tests, performed by E. J. Coulson of the Bureau of Agricultural and Industrial Chemistry, upon guinea pigs with a saline solution of a tomatin preparation (T-2<sup>3</sup>) containing 1.2 units/mg., have indicated that animals can tolerate the parenteral administration of as much as 10 mg. of such a preparation without fatal consequences, although unfavorable reactions are produced. The intravenous, intraperitoneal or subcutaneous injection of a solution containing 1 mg. of crude tomatin induced no notable reaction. However, the intravenous injection of 10 mg. into each of two animals produced immediate symptoms of distress and swelling of the injected legs. The animals recovered from the systemic reaction within 24 hours, but the injected legs remained swollen. Both animals continued to gain weight, and one recovered completely within 4 days; the second animal suffered necrosis of the injected leg. The intraperitoneal injection of 10 mg. of the tomatin preparation produced immediate and severe distress accompanied by a tensing of the abdomen. These symptoms subsided in 24 hours, and recovery was apparently complete. The subcutaneous injection of 10 mg. of the tomatin preparation induced a delayed systemic reaction from which the animal recovered within 24 hours. However, the cutaneous tissue at the site of the injection

formed a persistent ulcer which was not healed completely 10 days after injection.

It is impossible to ascertain from these preliminary tests whether purification of tomatin will result in increased or decreased toxicity. However, it is quite possible, in the light of recent results, that concentration of tomatin by alkali precipitation<sup>3</sup> also results in the concentration of toxic substances which do not appear to be associated with tomatin. More detailed toxicological tests will, of course, be made when the availability of purer tomatin preparations makes possible the administration of larger doses in terms of tomatin units.

#### THE PRESENCE OF TOMATIN-LIKE SUBSTANCES IN OTHER PLANTS

Extracts of plants other than the tomato have been found to inhibit cultures of *Fusarium*. Tomatin-like activity has been demonstrated in the autoclaved juice of the following plants: Sweet-potato plants (*Ipomoea batatas*) including the roots, potato tops (*Solanum tuberosum*) but not the tubers, pepper (*Capsicum frutescens*) plants and cabbage (*Brassica oleracea* var. *capitata*) leaves. It appears to be absent in lettuce (*Lactuca sativa*), jimson weed (*Datura stramonium*), white clover (*Trifolium repens*), dandelion (*Taraxacum officinale*), lima beans (*Phaseolus lunatus*).

#### SIGNIFICANCE OF PRELIMINARY RESULTS

The antibiotic, and particularly the fungistatic properties of tomatin are extremely interesting. Tomatin is by no means the only antibiotic agent that possesses fungistatic properties but most, if not all, of the known antibiotic agents are limited in their therapeutic usefulness because of their high toxicity. In view of the somewhat encouraging results of tests for toxicity reported here, and the marked *in vitro* effectiveness of tomatin against numerous pathogenic fungi, a complete investigation of the *in vivo* activity of this antibiotic agent is warranted and is in progress.

From the evidence presented it is obvious that tomatin, in its action toward microorganisms, is not highly specific. Likewise, it appears that this lack of specificity may also be characteristic of the substances responsible for the tomatin-like activity of the juices of certain other plants. These results may be of fundamental significance in the in-

vestigation of the biochemical factors responsible for disease resistance in plants.

#### ACKNOWLEDGMENTS

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#### SUMMARY

Directions for obtaining preparations of tomatin from tomato leaves, together with preliminary information concerning the chemical, physical and toxicological properties of tomatin preparations are presented. Tomatin has been found to be highly effective *in vitro* in inhibiting the human dermatophytic fungi, including species of *Trichophyton*, *Epidermophyton*, *Microsporum* and *Achorion*, and several of the fungi and yeast-like forms that cause internal disease, including species of *Monilia*, *Cryptococcus*, *Debaryomyces*, *Blastomyces*, *Coccidioides*, and *Histoplasma*. It is slightly effective against both gram-positive and gram-negative bacteria, species of the fungi *Fusarium*, *Penicillium*, *Aspergillus*, *Sporotrichum*, and *Monosporium* and the plant pathogenic *Actinomyces*. It is without effect upon the human pathogenic *Actinomyces* and the fungi responsible for the chromoblastomycoses. The presence of tomatin-like substances in certain plants other than the tomato is reported.

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# The Rate of Anaerobic Glycolysis of Various Hexoses in Mammalian Tissues \*

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## INTRODUCTION

The factors which control the rate of glycolysis in living tissues are not clearly understood. Some authors are inclined to attribute this rate solely to the concentration of the glycolytic enzymes concerned. Such an hypothesis would suggest a very high concentration in malignant tumors, embryonic or retina tissues (1), a somewhat smaller quantity in brain, testis or muscle, and a low concentration in kidney, liver, etc. This explanation neglects the possibility that the structures of the cells as well as unstable hormones may control the speed and keep it at a considerably lower rate than the mere concentration of the enzymes would permit. This was clearly shown some time ago in experiments on muscle tissue, where even cutting the muscle and suspending the tissue "brei" in phosphate increases the rate of resting glycolysis about ten times (2). By extraction of the enzymes with water, and addition of "Kochsaft" to raise the concentration of coenzymes to that in the living tissue, the rate of glycolysis is still further increased (3). More recent experiments on brain tissue (4, 5) gave the same results, i.e., water extract of brain, fortified with the various coenzymes, shows a rate of glycolysis,  $Q_{La}$  (mm.<sup>3</sup> CO<sub>2</sub> liberated by lactic acid/mg. dry weight of tissue/hr. at 38°C.) of 35-50 while the highest rate observed with slices of grey matter amounts to  $Q_{La}$  = 15-20.

It seemed worthwhile to investigate the conditions responsible for these changes making use of the different rates of glycolysis exhibited by different hexoses, especially glucose, fructose, and galactose.

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It was observed by O. Warburg (1) that tumor tissue glycolyzes fructose and galactose extremely slowly ( $Q_{L_a} = 3$  and  $1.3$ , respectively, instead of  $24$  as for glucose). This was also found by Loebel (6) as well as by Meyerhof and Lohmann (7) for brain tissue, although in this case fructose but not galactose was as readily oxidized as glucose.

According to Boyland (8), extracts of tumor glycolyze fructose and glucose at the same high speed, which is equal to the rate of glucose breakdown in slices of tumor. Moreover the great difference between glucose and fructose in tumor slices can be abolished by increasing the concentration of fructose. Ten *per cent* fructose instead of  $0.2\%$  gives a  $Q_{L_a}$  of  $30$ , which is nearly equal to that of glucose. Because of these findings, Boyland attributed the difference in glycolysis rate of the two sugars to different permeability through the cell membrane. When the diffusion gradient becomes sufficiently steep, the concentration of fructose in the interior of the cell would reach the level of maximal efficiency ( $0.2\%$ ) of glucose.

This explanation seems at first glance very convincing, and, as far as brain tissue is concerned, receives some support from the recent findings of Klein, Hurwitz and Olsen, that intravenously injected fructose diffuses more slowly than glucose from the capillaries into the tissue (9). Nevertheless, the different rate of diffusion cannot explain the equal rate of oxidation of the two sugars in the same tissue under the same conditions. For this and other reasons we reinvestigated the matter, using different methods of disintegration of the tissue. We hoped these studies would shed additional light on the general question of the controlling factors of glycolysis. After we had started the experiments with tumor tissue (transplanted rat sarcoma), we found that brain tissue of rats gave more reproducible values than the tumors at our disposal. Most of the work, therefore, was done on brain homogenates.

The experiments of Boyland on tumor slices could be confirmed in all details. Moreover, the same effect of concentration of fructose is found in slices of brain. However, his explanation cannot be entirely correct since the same difference of rate between glucose and fructose obtained after disintegration of the cells and also in this case is diminished and finally abolished by raising the concentration of fructose. For this demonstration we used the "homogenate" of brain, made according to Potter and Elvehjem (10).

An ample discussion in the literature is devoted to the question of whether the metabolism in homogenates equals that of tissue slices or tissue extracts. According to our findings, which seem to be in agreement with Potter's (11), the conditions of homogenization, the leeway of the homogenizer, the speed, time and temperature of homogenization, and the kind of suspending solution and dilution, determine which of

the extremes is approached or obtained. The results of Elliott and Henry indicate (12) that in their procedure the brain cells remain essentially intact and the coenzymes remain confined to the structural elements. But in the case of Utter *et al.* (5, 5a), who homogenize thoroughly in distilled water, the coenzymes are set free and destroyed by nucleotidases, while non-penetrating substances like hexosediphosphate are metabolized.

Although we homogenized normally in isotonic Ringer solution and maintained isotonic conditions during the incubation, the exchange of these cell substances was the same as that described by the latter authors. In part of our experiments the homogenate was frozen and thawed twice in a mixture of dry ice and alcohol, especially if the extract after centrifugation was to be used. This procedure increases the concentration of the dissolved enzymes. Otherwise we used the homogenate directly, after cooling but not freezing, in ice water.

## METHODS

In some of our experiments we used rat sarcoma 303, induced by methylcholanthrene by Mrs. Margaret Lewis at the Wistar Institute,<sup>1</sup> and which we transplanted from this strain.

Tumor tissue was used as slices only because the glycolysis of the homogenate from this tumor decreases very quickly and cannot be maintained at a high rate by addition of the known coenzymes or other "fortifying" substances. Brain tissue, on the other hand, was mainly used in the form of homogenates after preliminary experiments with slices had confirmed the older results (6, 7). The homogenate was either used as such or after centrifugation, in which case we call it "extract."

The homogenizer, blown by Mr. Nester, Swarthmore College, was fitted to a variable speed Cenco stirrer.

Details of the solutions used are given below, but one point may be mentioned here: if the homogenate is prepared in Ringer solution in the absence of bicarbonate and the salt added later, the glycolysis is appreciably lower than when bicarbonate is present from the start.

The following sugars were used: glucose—Merck dextrose c.p.; fructose from the Bureau of Standards or the Sugar Research Foundation<sup>2</sup>; galactose from the Paragon Test Laboratory, Orange, N. J.; mannose from Pfanstiehl; hexosediphosphate, purified from the barium salt made by the Schwarz Laboratories, New York. Hexosemonophosphate (Embsden ester) was prepared according to Lohmann (13).

Adenosinetriphosphate (ATP) was prepared as the barium salt according to Kerr with some modifications (14). Cozymase (DPN) was purified from a crude sample given us by Hoffmann-La Roche (*cf.* 15). Nicotinamide was the "Roche" preparation.<sup>3</sup>

<sup>1</sup> We are indebted to Mrs. Margaret Lewis for providing us with this tumor.

<sup>2</sup> We thank Dr. H. C. Hockett of the Sugar Research Foundation, Inc., for gifts of pure samples.

<sup>3</sup> We thank Hoffmann-La Roche, Inc., Nutley, N. J., for both preparations.



Formation of lactic acid as well as phosphoglyceric acid (in the presence of NaF) were usually measured manometrically, in the presence of bicarbonate, with  $N_2 + 5\%$   $CO_2$  in the gas space. In those systems in which the results were too greatly affected by other changes of acidity due to prevailing dephosphorylation or transphosphorylation, lactic acid was determined chemically, using the apparatus described by Lieb and Zacherl (16).

Phosphate was determined by the method of Fiske and SubbaRow (17) using the modification of Lohmann and Jendrassik (18). We used an Evelyn photoelectric colorimeter with the filter of 660  $m\mu$ .

## 1. GLYCOLYSIS IN TISSUE SLICES

Fig. 1 and Table I exemplify the rates of glycolysis in sarcoma slices with different sugars. They confirm the findings of Boyland. With 2% and 5% fructose the rate of glycolysis of glucose is reached. Fig. 1 shows, moreover, that galactose behaves similarly, although only 50% of the rate of glucose is attained. Table I shows, in addition, that mixtures of fructose and glucose give only a slightly higher rate than that obtained *in maximo* either with glucose or fructose alone (Expt. 90).

TABLE I (GL. 90)  
 $Q_{La}$  of Slices of Sarcoma

	0.2% glucose	Fructose			Mixture of 0.2% glucose and fructose		
		0.2%	2.0%	5.0%	0.2%	2.0%	5.0%
1 hour	30.3	8.0	33.7	34.2	29.5	36.1	39.4
2 hours	29.6	6.6	30.2	28.0	25.6	34.0	34.0

Brain slices (grey matter) give similar results although somewhat less regular.  $Q_{La}$  values in one hour experiments vary for 0.2% glucose from 7.5 to 13; 0.2% fructose from 2.2 to 7.5; 0.2% galactose from 2.5 to 3.5. Higher concentrations of fructose and galactose give higher  $Q$  values with considerable variations.

## 2. GLYCOLYSIS IN HOMOGENATES AND EXTRACTS FROM BRAIN

As stated above, homogenates from brain exhibiting very different metabolic features can be obtained by slight variations in technique. If the homogenate is made in distilled water, the remaining structures are "inactive." In such a case, as shown by Utter *et al.* (5) and confirmed

by us, the whole glycolytic system passes into solution. The homogenate, the opalescent centrifuged extract, or the mixture of this extract with the particles, give generally the same values for  $Q_{La}$ , *ca.* 40–60, provided all coenzyme factors are added in an amount corresponding to the concentration in whole brain.

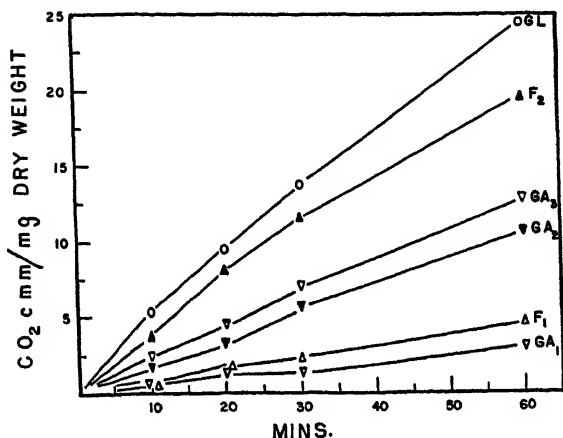


FIG. 1. Glycolysis in Slices of Transplanted Sarcoma (Gl. 32). Vol. 2 cc.; dry weight 3–6 mg.; 5% CO<sub>2</sub> in N<sub>2</sub>.

○ Gl. 4 mg. glucose, △ F<sub>1</sub> 4 mg. fructose, ▲ F<sub>2</sub> 190 mg. fructose. ▽ GA<sub>1</sub> 4 mg. galactose, ▼ GA<sub>2</sub> 48 mg. galactose, ▽ GA<sub>3</sub> 96 mg. galactose.

We may add to the findings of these authors, that in such systems fructose, even in low concentration, gives the same or a somewhat higher rate of glycolysis than glucose. We call this the extract-rate. On the other hand, galactose, even in high concentration, shows hardly any activity at all.

To determine the conditions causing the normal rate of glycolysis of slices, we homogenized in Ca-free Ringer solution with addition of Mg. Due to thorough homogenization the cells are destroyed and the coenzymes pass into solution, where they are decomposed by nucleotidases and phosphatases. Upon addition of the coenzyme factors a great difference between the homogenate and the centrifuged extract appears. The complete homogenate shows a low rate of glycolysis with a great difference between glucose on one hand and fructose and galac-

tose on the other, a difference which is decreased by higher concentrations of the latter sugars. If the particles are removed by centrifugation, the fortified extract gives the extract rate of glycolysis, where glycolysis of fructose generally surpasses glucose, and galactose is practically stable.

Actually, these extract rates are only slightly increased when the homogenized brain is frozen and thawed several times, using dry ice, or if the homogenate is made with distilled water according to Utter *et al.* (*cf.* also 5a).

The homogenate was made either with 3 or 5 parts of Ringer solution in which the  $\text{CaCl}_2$  is replaced by  $\text{MgSO}_4$ <sup>4</sup> and containing 28% of its volume of isotonic  $\text{NaHCO}_3$  solution. In the Ringer solution enough nicotinamide was dissolved to give a final concentration, after addition of all components, of 0.3–0.6%. This concentration checks the decomposition of cozymase (DPN) according to Mann and Quastel (19) and Handler and Klein (20) (*cf.* the following pages). The total volume was between 0.8 and 1.2 cc., where, according to the special purpose, either 0.2 cc. of the concentrated homogenate or extract or 0.5 cc. of the diluted homogenate was used. In a completely "fortified" system 0.05 cc. of hexosediphosphate (containing 25  $\gamma$  P) were added and, 2 minutes before starting the readings, a mixture of 0.05 cc. cozymase (0.3 mg. pure DPN), and 0.05–0.15 cc. ATP containing 20–50  $\gamma$  pyrophosphate P and 0.05 cc. bicarbonate was tipped in from a side bulb. In some series 0.1 cc. glutathione (0.1 *N*) and 0.1 cc. of 0.1 *N* phosphate mixture of pH 7.2 were also added. Inorganic phosphate increases the rate of glycolysis appreciably, especially with low concentrations of ATP.

A typical experiment in which the activity of the complete homogenate was compared with that of the centrifuged extract of the same homogenate is reproduced in Fig. 2 (No. 119). The numbering of the ordinates in this and all following figures refers to the amount of  $\text{CO}_2$  liberated/mg. of dry weight of the homogenate, from which the preparation was made, regardless of whether the total homogenate was taken or the extract, which contains only a part of its dry weight. As Fig. 2 shows, glucose and fructose react in the first 30 minutes 7 times as fast in the extract as glucose does in the homogenate, and, moreover, fructose reacts in the homogenate only one-third as fast as glucose. The  $Q_{L_{\infty}}$  values, 10.7 for glucose, 3.9 for fructose, are similar to

<sup>4</sup> The modified Ringer solution was mixed from isotonic solutions of  $\text{NaCl}$ ,  $\text{KCl}$ , and  $\text{MgSO}_4$  in the proportions: 10 cc. of 0.9%  $\text{NaCl}$ , 0.5 cc. of 1.15%  $\text{KCl}$ , 0.4 cc. of 3.82%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . To 8 cc. of this solution were added 3.2 cc. of 1.3%  $\text{NaHCO}_3$ . If the homogenate was made with 3 parts of solution, Ringer and bicarbonate were mixed 1:1.

those of slices, while the  $Q_{La}$  values of the extract, about 70, are much larger.

If the cell particles are centrifuged, washed with Ringer solution, and added to the extract, they lower the extract rate to that of the original homogenate. This is shown in Fig. 3. A similar inhibition by the structural elements has already been observed by Geiger (4). The composition of the samples was similar to that of Fig. 2.

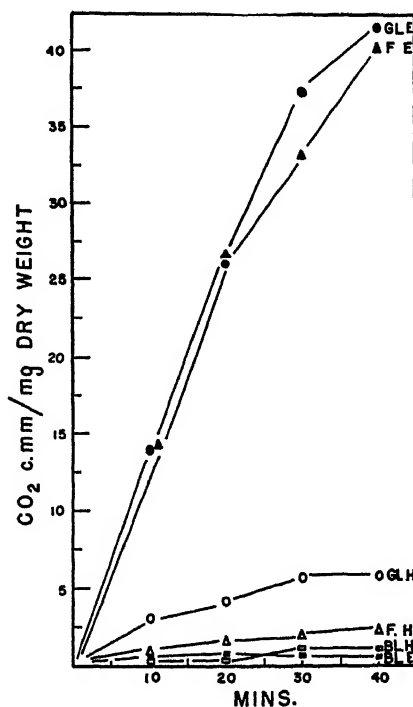


FIG. 2. Glycolysis in Complete Homogenate (H) and, after Centrifuging, in Extract (E). Homogenate frozen and thawed (Gl. 119). Vol. 0.75 cc. containing 0.2 cc. homogenate or extract (1:3) with Ca-free Ringer, bicarbonate and  $MgSO_4$ , nicotinamide to 0.6% final concentration. 0.05 cc. hexosediphosphate (20  $\gamma$  P); 2 minutes before start 0.05 cc. (0.3 mg. DPN), 0.05 cc. ATP (30  $\gamma$  7-min. P) 0.05 cc. isoton. bicarbonate tipped in.  $\square$  ■ Bl. E and H. Blank of extract and homogenate. ● Gl. E. and ○ Gl. H. 3 mg. glucose in extract and in homogenate. ▲ F. E. and △ F. H. 3 mg. fructose in extract and in homogenate.

In respect to sugar concentration the complete homogenate behaves like the tissue slices. While the extract rate is not changed by increasing sugar concentration (it is sometimes even lowered), the rate in the

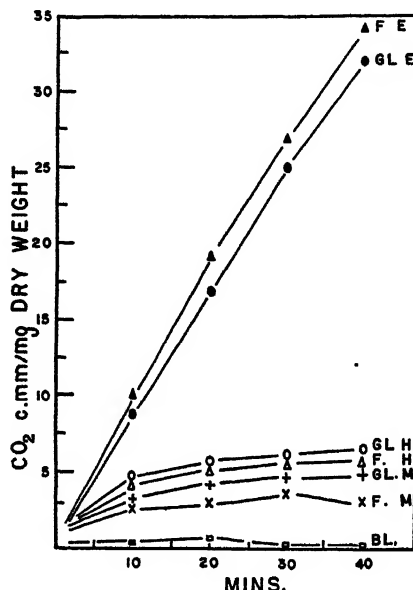


FIG. 3. Glycolysis in Complete Homogenate (H), in Extract (E), and in Mixture of Extract and Washed Particles (M) (GL 122). Vol. 0.85 cc. containing 0.2 cc. homogenate or 0.15 cc. extract or 0.15 cc. extract + 0.2 cc. washed particles. Other additions as in Fig. 2.  $\square$  Blank of extract.  $\bullet$  GL. E,  $\circ$  H. and  $+$  M. 3 mg. glucose in extract, in homogenate and in mixture.  $\blacktriangle$  F. E. and  $\triangle$  H. and  $\times$  M. 3 mg. fructose in extract, in homogenate and in mixture.

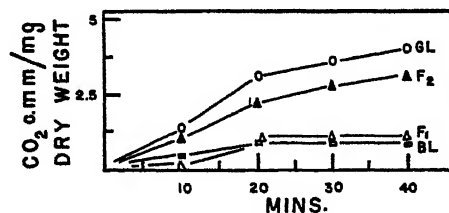


FIG. 4. Glycolysis in Homogenate, not Frozen. (GL 105). Vol. 0.9 cc. containing 0.55 cc. homogenate (1:5). Nicotinamide of 0.3% final concentration. 2 minutes before start 0.075 cc. cozymase (0.5 mg.) tipped in.  $\square$  BL. blank.  $\circ$  GL. 2.5 mg. glucose.  $\triangle$  F<sub>1</sub> 2.5 mg. fructose.  $\blacktriangle$  F<sub>2</sub> 10 and 20 mg. fructose (identical curves).

homogenate is increased with 2-5% of fructose and galactose. This is shown for one experiment each with fructose and galactose in Figs. 4 and 5. One *per cent* of these sugars gives intermediate values (not shown in the Figs.).

In the experiments reproduced here (Gl. 105 and 106) the homogenate was not frozen and thawed, and no ATP but only cozymase was added. Under these conditions the decline of glycolysis is somewhat less and the difference between the sugars appears more marked. While in these cases fructose and galactose behave similarly, this is not so in extracts. The contrast is shown in Fig. 6 as compared with Figs. 2 and 3. While glucose gives the usual high  $Q_{La}$  of 45, high and low concentrations of galactose give nearly zero. The blank value must be subtracted.

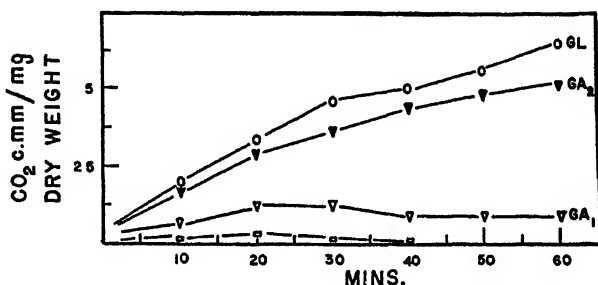


FIG. 5. Glycolysis in Homogenate as in Fig. 4 (Gl. 106).  $\square$  Bl. Blank.  $\circ$  GL. 2.5 mg. glucose.  $\nabla$  Ga<sub>1</sub> 2.5 mg. galactose,  $\blacktriangledown$  Ga<sub>2</sub> 20 mg. galactose.

(The relatively high reading for 5 minutes, both in the blank and the galactose samples is caused by splitting of ATP.) In this experiment 0.1 cc. ATP with 45  $\gamma$  pyrophosphoric-P was added at the beginning.

Experiments of the type depicted in Figs. 4 and 5 were repeated many times with nearly the same results, when the conditions were either the same or slightly modified. In some cases quartz powder was added during homogenization to insure destruction of the cells. This did not change the outcome although the decline was somewhat more rapid. Data of such an experiment are recorded in Table II (Exp. 109). Regarding other sugars it may be stated that with mannose the same rates are obtained as with glucose, both in homogenates as well as in extracts. Hexosediphosphate and hexosemonophosphate are rapidly split in homogenates and in extracts. In both cases the turnover stops approximately at the time the added hexose ester is completely split

TABLE II

*Mm.<sup>3</sup> CO<sub>2</sub> Formed with 0.5 cc. Homogenate (1:5) (+ Quartz Powder)*

Add	0	Glucose	Fructose		Galactose	
		0.25%	0.28%	1.4%	0.25%	1.4%
First 30 min.	1.5	47.5	2	27	10	33
Second 30 min.	0	23.5	2	18	0	9

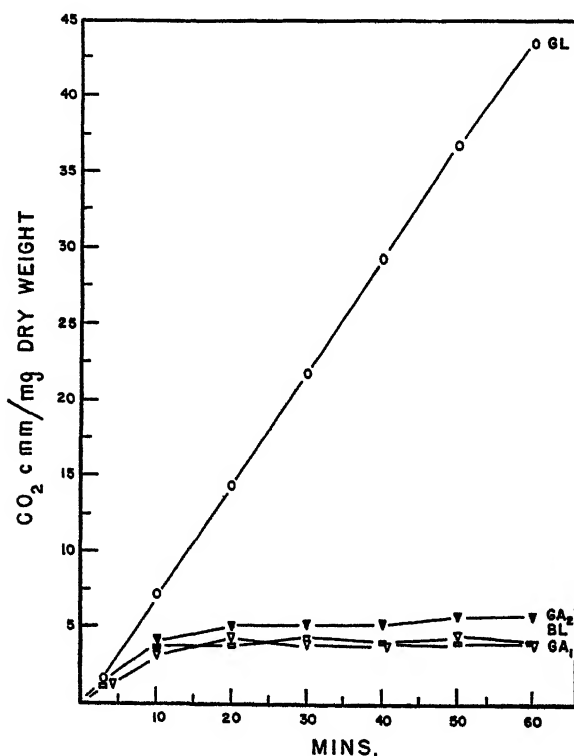


FIG. 6. Glycolysis in Centrifuged Extract. 0.2 cc. extract (1:3) in total volume of 0.8 cc., nicotinamide in final concentration of 0.55%. 0.1 cc. *M*/10 phosphate. 0.05 cc. cozymase (0.3 mg.) + 0.1 cc. ATP (60  $\gamma$  7-min. P) + 0.05 cc. bicarbonate tipped in at beginning. CO<sub>2</sub> not corrected for retention (Gl. 146).  $\square$  BL. blank.  $\circ$  GL. 4 mg. glucose.  $\nabla$  GA<sub>1</sub> 4 mg. galactose,  $\blacktriangledown$  GA<sub>2</sub> 10 mg. galactose.

to lactic acid and phosphate. Account must be taken here of the retention of  $\text{CO}_2$  which amounts to 1.25 with hexosediphosphate and to somewhat less with the monophosphate. It is of some interest that in the centrifuged extract the initial rate of turnover of hexosemonophosphate is higher ( $Q_{L_1} = 56$ ) than that of hexosediphosphate ( $Q_{L_1} = 36$ ) while in the homogenate the reverse is the case (Fig. 7.) As was shown formerly (21), the speed of decomposition of hexosediphosphate in the absence of P-acceptors is conditioned by the concentration of

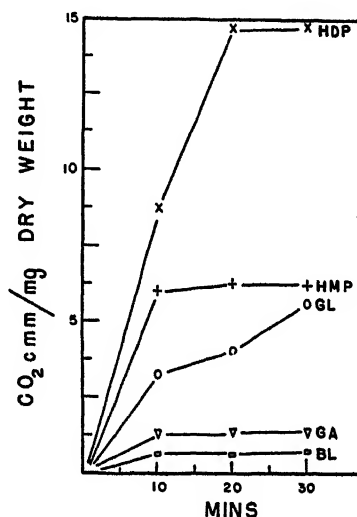


FIG. 7. Glycolysis in Homogenate, not Frozen. Each sample 0.5 cc. homogenate (1:5) in 1.15 cc. total volume, 0.25% final concentration of nicotinamide (Gl. 151). 0.05 cc. cozymase (0.3 mg.), 0.1 cc. ATP (60  $\gamma$  7-min. P), 0.1 cc. bicarbonate tipped in 2 minutes before start.  $\square$  Bl. blank.  $\circ$  Gl. 2 mg. glucose.  $\nabla$  Ga 2 mg. galactose.  $\times$  HDP. 0.7 mg. P of hexosediphosphate (= 2 mg. hexose) + HMP. 0.13 mg. P of hexosemonophosphate (= 0.76 mg. hexose).

adenylypyrophosphatase ("apyrase"). This indicates, in agreement with the following data, that the concentration of this enzyme is higher in the homogenate than in the extract. The turnover of hexosediphosphate in the homogenate is an additional proof that it does not contain impermeable cell interfaces (see also (21a)).

The decline of the turnover in the homogenate is in great part due to the decomposition of the coenzymes. This can be ascertained by



repeating their addition after 30 minutes (see Fig. 8). A new increase of the rate follows after this addition (arrow in Fig. 8). This extra acid is mainly lactic acid and only partly due to the splitting of ATP, as was demonstrated by chemical determinations. Moreover, the splitting of the total ATP to adenylic acid and phosphoric acid could only give 30 mm.<sup>3</sup> of acid, while, in the samples containing sugar, between 60 and 80 mm.<sup>3</sup> are formed in the first 10 minutes after the second addition of the coenzymes.

The rate of glycolysis indicating the typical difference between glucose and fructose is only obtained with Ringer-homogenate. Homogenate with distilled water gives the same turnover rate as extract,

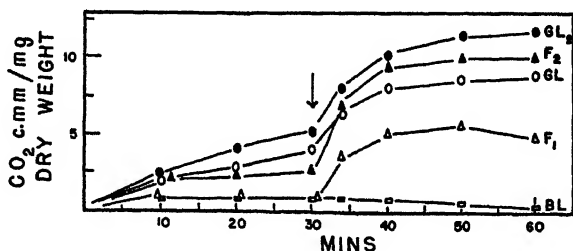


FIG. 8. Glycolysis in Homogenate, not Frozen (GL 131). 0.55 cc. homogenate (1:5) in final volume of 1.25 cc., nicotinamide 0.75% final concentration. Mixture of 0.05 cc. cozymase (0.3 mg.) + 0.1 cc. ATP (50  $\gamma$  7-min. P) + 0.05 cc. bicarbonate tipped in 2 minutes before start and again after 30 minutes (arrow). □ Bl. blank. ○ GL<sub>2</sub> glucose 2 and 10 mg. (identical curves), ● GL<sub>10</sub> glucose 10 mg. + 0.1 cc. *M*/10 phosphate. △ F<sub>1</sub> 2 mg. fructose, ▲ F<sub>2</sub> 10 mg. fructose + 0.1 cc. *M*/10 phosphate.

with fructose slightly superior to glucose. For instance, the  $Q_{L_{30}}$  (for 30 minutes) of water-homogenate with 0.35% glucose is 35, with 0.35% fructose 56. An extract from this homogenate with 0.35% glucose gives  $Q_{L_{30}} = 56$ , with 0.35% fructose, 61. This confirms the results of Utter *et al.* (5). If NaCl and KCl are left out, but the usual amount of bicarbonate, MgSO<sub>4</sub> and nicotinamide are added for preparing the homogenate, intermediate values are obtained, where the homogenate rate is much lower than the extract rate, but the difference between glucose and fructose is less marked.

### 3. DESTRUCTION OF COZYMASE

The decline in the speed of glycolysis as recorded in Fig. 8 and its restoration by addition of cozymase and ATP are, in the main, due to

ATP and not to cozymase. The regeneration of ATP during the glycolytic cycle is insufficient to keep pace with the dephosphorylation caused by the high rate of activity of apyrase and possibly of unspecific phosphatases. But apyrase is strongly adsorbed by the structural elements (*cf.* 21).

On the other hand, direct determination of the destruction of cozymase in these glycolytic experiments reveals that, due to the presence of nicotinamide, only  $\frac{1}{2}$ – $\frac{2}{3}$  of the added cozymase is decomposed during 50 minutes, nearly as much in homogenate as in extract. The remaining amount is sufficient to ensure nearly maximal speed of glycolysis. For these experiments we used the "apozymase test" of H. von Euler (22), in the form described earlier (23) (*cf.* Table III).

TABLE III  
*Cozymase Destruction in Glycolytic Samples*

No.	Preparation	Nicotinamide	Incubation	Added sugar	Per cent cozymase left at end of incubation	
					Expt. I	Expt. II
159	Homogenate 1:5	<i>per cent</i> 0.3	<i>min.</i> 20	4 mg. glucose	76	
		0.3	50	4 mg. glucose	61	
160	Homogenate 1:5	0.3	50	Ringer	19	47
161		0.3	50	4 mg. glucose	23	49
		0.3	50	4 mg. fructose	25	55
162	Extract 1:3	0.36	50	Ringer	35	
		0.36	50	4 mg. glucose	37	
		0.36	50	4 mg. fructose	35	

Samples similar to those of Figs. 4 and 5 served for these tests. The final concentration of nicotinamide was 0.3%. The enzymes were inactivated by dipping the test tube containing the sample into boiling water for 3 minutes. The suspension was centrifuged after cooling. Aliquots of the supernatant were added to the apozymase suspension. Apozymase was prepared according to Ohlmeyer (23) from Fleischmann dry yeast No. 2040.<sup>5</sup> Ninety mg. of washed yeast were used in 2 cc. total volume of

<sup>5</sup> We thank Dr. C. N. Frey from Fleischmann Laboratories, New York, for a sample of the dry yeast.

fluid in manometric vessels of 15 cc. capacity. One-tenth cc. ATP was added to each sample to eliminate a possible disturbance by variation of the ATP content of the glycolytic setups.

Although the results are somewhat variable, the destruction of cozymase does not differ as between Ringer, glucose or fructose samples, nor between homogenate and extract. The amount of the added 0.3 mg. cozymase which remains/0.9 cc. after 50 minutes would be sufficient to maintain the initial rate of glycolysis.

#### 4. THE DEPHOSPHORYLATION AND TRANSPHOSPHORYLATION OF ATP

The decomposition of ATP was measured: (a) in samples corresponding to those described in section 2, (b) in the presence of  $N/10$  NaF or of NaF plus iodoacetate or iodoacetamide to inhibit the redox step.

##### *a. The Turnover of ATP in Glycolytic Samples*

In Table IV the values of the phosphate analyses at the end of the manometric experiments are compared with the initial values. The  $Q_{La}$  values are calculated for 30 minutes. Two minutes before starting the manometric readings 0.15 cc. ATP containing 120  $\gamma$  of 7-min. P was tipped in together with 0.05 cc. cozymase (0.3 mg.). Since some more ATP splits after the lapse of 2 minutes, a more accurate  $Q_{La}$  value is obtained by subtracting the  $Q_{La}$  value of the blank. This difference is given in heavy type. One-half cc. of homogenate (1:5) or 0.3 cc. of extract (1:3) were used.

TABLE IV  
*Phosphate Turnover in Glycolyzing Brain Preparations*

Preparation	Min.	Sugar	Mg.	$Q_{La}$	$Q_{La}$ (corr.)	Pyro P present	Inorg. P change	7-min. P change
Homogenate (164) (1:5) 0.5 cc.	0	—	—	—	—	$\gamma$ 128	$\gamma$ —	$\gamma$ —
	32	—	—	3.1	—	(23)	+188	+86
	32	Glucose	4	11.7	8.6	(18)	+157	+48
	32	Fructose	4	6.5	3.4	(25)	+165	+62
	32	Fructose	20	9.8	6.7	(26)	+161	+60
Extract (165) (1:3) 0.3 cc.	0	—	—	—	—	130	—	—
	32	—	—	5.9	—	15	+168	+52
	32	Glucose	4	52.5	46.6	55	+39	-36
	32	Fructose	4	72.5	66.6	66	0	-69
	32	Fructose	20	65.7	59.8	72	0	-63

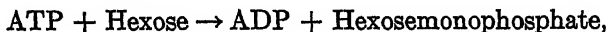
The changes in phosphate reflect quite clearly the glycolytic activity. In the extract (Gl. 165) ATP is dephosphorylated completely only in the blank. Actually more inorganic phosphate appears than the pyro P present at the start, 52  $\gamma$  P deriving from other sources. With sugar very little dephosphorylation occurs. The transphosphorylation with fructose surpasses that with glucose. At the same time the  $Q_{La}$  values with fructose are nearly 40% greater than with glucose. No inorganic phosphate is split off with fructose, but a quarter of the available P is split with glucose.

In the complete homogenate (Gl. 164), on the other hand, all of the ATP and part of the preformed phosphate esters are split. The remaining 7-min. P is so small that it is questionable if it corresponds to the remainder of the ATP. Here also the increase of inorganic phosphate is slightly higher in the blank than in the samples containing sugar.

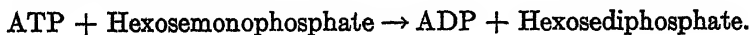
In such experiments, however, the cause and the effect are not clearly differentiated. Only that tendency is evident, that the higher the glycolysis the more transphosphorylation prevails, and the lower it is, the more dephosphorylation prevails. A clearer insight is gained when glycolysis is inhibited by NaF or by a combination of NaF plus iodoacetate or iodoacetamide.

#### *b. Turnover of ATP in the Presence of NaF*

NaF partly inhibits the apyrase. If iodoacetate is also added, no energy-yielding reactions can take place which regenerate ATP. Nevertheless, splitting off of inorganic phosphate is greater in the blank than in the samples with sugar. In the latter case the greater part of the ATP is transphosphorylated by the hexokinase (24) according to the equation:



and the subsequent step



These reactions do not easily proceed further to adenylic acid, since apparently the myokinase of Kalckar (25) or ADP-dismutase is not active in our system. The figures presented in Table IV indicate that the irreversible split of ATP must be made responsible for the decline

of glycolysis in the homogenate compared with the extract. On the other hand, in the presence of NaF and iodoacetate (Table V) fructose is as easily phosphorylated by ATP as glucose both in homogenates and in extracts. There is no difference between high and low concentrations of fructose. Fructose, mannose, and glucose exhibit about the same reactivity in the presence of hexokinase and protect ATP in the same way against dephosphorylation. Only galactose is an exception. It reacts very slightly, but somewhat more in higher concentration. It also reacts less in extract than in homogenate, in accordance with its behavior in glycolysis. In the presence of fluoride alone, without

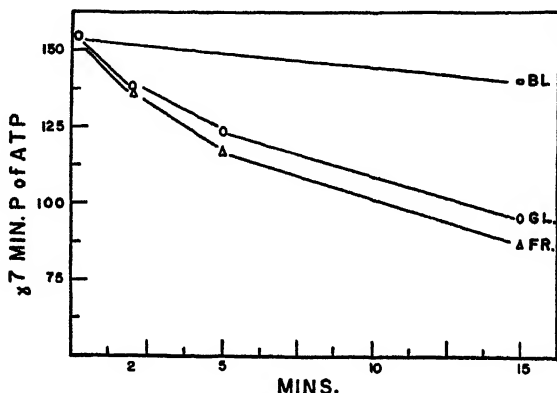


FIG. 9. Transphosphorylation with ATP in Extract. 0.6 cc. extract (1:5) in 1.3 cc. total volume  $N/12$  NaF. Decrease of 7-min. P without change of inorganic phosphate corresponds to hexokinase reaction.  $\square$  BL. blank.  $\circ$  GL. 4 mg. glucose.  $\triangle$  FR. 4 mg. fructose (Exps. 195/196).

inhibiting the redox action, no inorganic phosphate is split with glucose and maximum transphosphorylation is obtained after 5 minutes. With galactose, transphosphorylation is weak and in the blank practically absent (Gl. 203).

When redox action is inhibited by iodoacetate or iodoacetamide, inorganic phosphate is split off somewhat more in homogenates than in extracts, but transphosphorylation is about the same in both cases.

The progress of transphosphorylation in an extract is depicted in Fig. 9.

In Table V the pyrophosphate P present, calculated from the difference: 7-min. P minus inorganic P is, at the end of the experiment,

TABLE V

*Transphosphorylation and Dephosphorylation in Presence of  $8 \times 10^{-2}$  M NaF*

No.	Preparation	SH-inhibitor	Time of incubation	Sugar	Mg.	Pyro P present	Increase of inorg. P	Change of 7-min. P
			<i>min.</i>			$\gamma$	$\gamma$	$\gamma$
203	Extract 1:5 0.6 cc.	—	0	—	—	146	—	—
			15	—	—	105	35	-4
			5	Glucose	4	93	0	-59
			15	Glucose	4	83	5	-59
			5	Galactose	4	121	11	-15
			15	Galactose	4	109	28	-10
134	Homogenate 1:5 0.5 cc.	$5 \times 10^{-3}$ Iodo- acetate	0	—	—	212	—	—
			15	—	—	144	77	+8
			5	Glucose	4	140	57	-16
			15	Glucose	4	118	57	-39
			5	Fructose	4	98	80	-34
134a	Extract 1:5 0.5 cc.	$5 \times 10^{-3}$ Iodo- acetate	0	Glucose	—	205	—	—
			5	Glucose	—	165	28	-11
			15	Glucose	—	131	28	-45
			15	Fructose	—	125	30	-45
142	Homogenate 1:5.5 0.5 cc.	$5 \times 10^{-3}$ Iodoacet- amide	0	—	—	214	—	—
			15	—	—	122	100	+4
			15	Glucose	4	132	50	-34
			15	Fructose	4	104	56	-56
			15	Fructose	20	110	63	-34
			15	Galactose	4	134	73	-6
			15	Galactose	20	122	68	-23
145	Extract 1:5.5 0.5 cc.	$5 \times 10^{-3}$ Iodo- acetate	0	—	—	175	—	—
			15	—	—	165	43	+33
			15	Glucose	4	120	24	-30
			15	Fructose	4	117	18	-43
			15	Fructose	20	132	12	-30
			15	Galactose	4	153	47	+27
			15	Galactose	20	153	31	+10
155	Extract 1:5.5 0.5 cc.	$5 \times 10^{-3}$ Iodo- acetate	0	—	—	280	—	—
			15	—	—	254	33	+6
			5	Glucose	4	232	12	-36
			15	Glucose	4	206	13	-66
			5	Mannose	4	234	15	-31
			15	Mannose	4	200	16	-67

mainly ADP which is practically stable in our enzymatic system. (ADP-P = half the initial value of pyro-P). The hexokinase activity is measured by the decrease of 7-min. P (last column). The ATPase activity is measured by the increase of inorganic P. If simultaneously the 7-min. P increases, the corresponding increase of inorganic phosphate is caused by other esters than ATP.

These figures, as far as the ATPase (or "Apyrase") is concerned, may be compared with the maximal activity of this enzyme in rat brain as found by Dubois and Potter (26). According to these authors 1 mg. of fresh tissue splits 7.0  $\gamma$  of 7-min. P in 15 min. at 38°C. in the presence of the optimal  $\text{Ca}^{++}$  concentration ( $8 \times 10^{-4}$  mole), and 2.4  $\gamma$  7-min. P in the absence of  $\text{Ca}^{++}$ . For the average amount of tissue used in the experiments of Tables IV and V (about 75 mg. fresh tissue) this would mean 530  $\gamma$  P *in maximo*, or, since no  $\text{Ca}^{++}$  was added, at least 180  $\gamma$  P. This latter amount is about the total amount of ATP added in our experiments. A comparison seems possible only for the blank where transphosphorylation is avoided and no ATP can be regenerated during glycolysis. While the experiments recorded in Table IV cannot be evaluated because all of the ATP is split before the end of the incubation, the amounts decomposed, as presented in Table V are much less, 80-96  $\gamma$  P in the complete homogenates (134 and 142). This must be attributed to the fluoride inhibition of apyrase.

##### 5. LACTIC ACID FORMATION WITH DIFFERENT DILUTIONS OF HOMOGENATE AND ATP

From the foregoing, several new problems arose which can be investigated experimentally:

a. If the main reason for the low glycolysis in homogenates compared with extract is the decomposition of ATP owing to the high concentration of apyrase, then by adding more ATP, especially repeated additions during short intervals, to the homogenate, the rate should rise to the rate of glycolysis in extracts.

b. If the "controlling influence" of the structural elements inhibits the breakdown of fructose more than that of glucose, then a 'dilution of the homogenate should be found, where the difference disappears because the particles become too few to suppress the extract rate of glycolysis of fructose.

c. Because transphosphorylation of "physiological" and still higher concentrations of ATP proceeds in homogenates equally well with fructose or glucose, as shown by the data presented in Table V, while the ATP concentration is normally lowered by the apyrase to much smaller values, the possibility should be envisaged that the affinity for low concentrations of ATP is less with fructose than with glucose.

The determinations for answering these questions were mostly made by chemical analyses of lactic acid because of the acid formation during the splitting of ATP. The good agreement of the chemical and manometric determinations of lactic acid formation in strongly glycolyzing extracts may be seen from the examples recorded in Table VI.

TABLE VI

*Chemical and Manometric Determination of Lactic Acid in Extract*  
0.2 cc. extract in 0.75 cc. total, 1 hour. (No. 126)

Additions	Mm. <sup>4</sup> CO <sub>2</sub>	Initial l. a. mg.	Final l. a. mg.	Chem. l. a. formed mg.	Manom. l. a. formed mg.	Q <sub>La</sub> for 1 hr. (manom.)
(1) 4 mg. Glucose +0.1 cc. ATP+DPN	370	.15	1.53	1.38	1.48	62
(2) 4 mg. Glucose +0.15 cc. ATP+DPN	340	.15	1.40	1.25	1.33	68
(3) 4 mg. Glucose +0.1 cc. ATP+DPN +0.1 cc. phosph.	430 <sup>*</sup>	.15	1.90	1.75	1.72	71

<sup>\*</sup> Retention of CO<sub>2</sub> = 1.15.

In Table VII a comparison is made between repeated additions of ATP—a 40–50  $\gamma$  portion of 7-min. P every 5 or 2 minutes—and a single addition of 80–100  $\gamma$  7-min. P at the beginning, for incubations of 15–40 minutes. The amount of lactic acid formed after repeated additions is greater and the decline of the turnover with fructose is smaller. With additions every 5 minutes the differences between glucose and fructose do not disappear. As can be seen in experiment 170 they are still similar to those found in the manometric experiments of Section 2. If, however, the interval between the additions of ATP is further reduced and 50  $\gamma$  of 7-min. P is added every 2 minutes over periods of 15 and 30 minutes (Experiment 235) the extract rate is



TABLE VII

*Formation of Lactic Acid in Homogenates after Single and Repeated Additions of ATP*  
1.0 cc. homogenate per sample (1:5)

No.	Time	Sugar	Mg.	Total ATP after repeated additions 7-min. P		ATP with 1 add. 7-min. P	Formation of lactic acid		Q <sub>La</sub> calc.	
					Intervals		After repeated add.	After 1 add.	After repeated add.	After 1 add.
	<i>min</i>			$\gamma$	<i>min.</i>	$\gamma$	$\gamma$	$\gamma$		
167	40	—	—	320	5	—	39.6	—	0.4	—
	40	Glucose	4	320	5	80	555	387	6.0	4.2
	40	Fructose	4	320	5	80	336	238	3.6	2.6
169	40	—	—	320	5	—	83	—	0.87	—
	20	Glucose	4	160	5	—	650	—	13.9	—
	40	Glucose	4	320	5	80	1040	853	11.1	9.1
	20	Fructose	4	160	5	—	141	—	3.0	—
	40	Fructose	4	320	5	80	330	16	3.5	.2
	40	—	—	320	5	—	133	—	1.5	—
170	40	Glucose	4	320	5	—	1090	—	12.3	—
	40	Fructose	4	320	5	—	306	—	3.45	—
	40	Fructose	20	320	5	—	572	—	6.4	—
	40	Galactose	4	320	5	—	100	—	1.1	—
	40	Galactose	20	320	5	—	248	—	2.8	—
	40	—	—	—	5	80	—	129	—	1.31
168	20	Glucose	4	—	5	80	—	351	—	7.5
	40	Glucose	4	—	5	80	—	587	—	6.3
	20	Fructose	4	—	5	80	—	239	—	5.0
	40	Fructose	4	—	5	80	—	204	—	2.2
	40	—	—	—	5	80	—	—	—	—
235	30	—	—	—	2	100	52	52	0.9	0.9
	15	Glucose	4	450	2	—	609	—	21.8	—
	30	Glucose	4	800	2	100	1150	504	20.3	9.6
	15	Fructose	4	450	2	—	429	—	15.4	—
	30	Fructose	4	800	2	100	1190	178	21.2	3.2
	30	—	—	—	2	100	—	—	—	—

\* 0.8 cc. homogenate.

approached. Furthermore, the rates of glycolysis of fructose and glucose become nearly identical, the latter corresponding to Q<sub>La</sub> 21.8 and 20.3, the former to Q<sub>La</sub> 15.4 and 21. In the same experiment, with only one addition of ATP, the Q<sub>La</sub> for glucose is 9.6 and for fructose 3.2, a

proportion of 3:1. This is a clear proof that the concentration of ATP, continuously lowered by the action of the apyrase, controls not only the absolute rates of glycolysis in the homogenates but also the difference of the turnover of glucose and fructose.

As could be expected, a critical dilution of the homogenate exists where the influence of the particles with the adsorbed enzymes is sufficiently marked to keep the glycolysis rate down and repress that of fructose more than that of glucose. If this dilution is exceeded, both are metabolized with the same speed. This result is demonstrated in

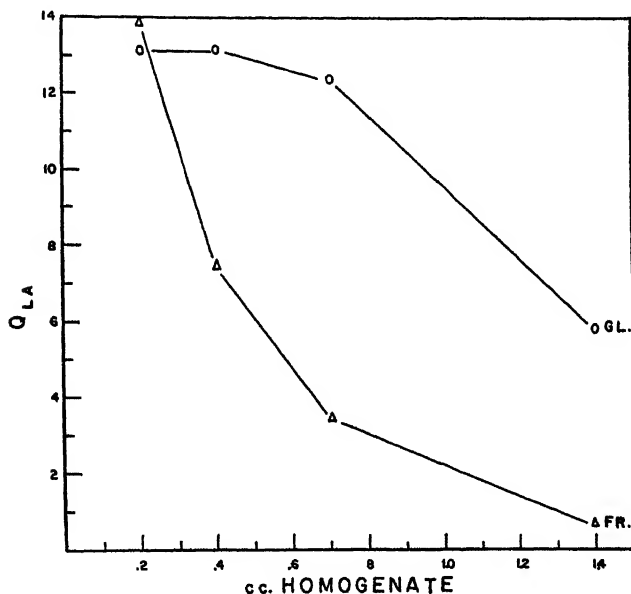


FIG. 10. Lactic Acid Formation with Different Dilutions of Homogenate (chem. determinations) (No. 171). 40 minute incubation. Abscissa cc. homogenate (1:5) in 2 cc. total vol. Ordinate  $Q_{LA}$  calc. (0.9 mg. = 224 mm.<sup>3</sup> CO<sub>2</sub>). Addition of 0.1 cc. co-enzyme (0.6 mg.) and ATP (40 γ 7-min. P) for 0.2 and 0.4 cc. homogenate, and 80 γ 7-min. P for 0.7 and 1.4 cc. homogenate. O Gl. 4 mg. glucose. Δ Fr. 4 mg. fructose.

Fig. 10. Incubation time was 40 minutes and lactic acid was determined chemically, but is recalculated as  $Q_{LA}$ . Only for the highest dilution. 0.2 cc. homogenate in 2.0 cc. total volume, is  $Q_{LA}$  for fructose the same as for glucose (14) while this value remains nearly constant for glucose up to 0.7 cc. homogenate in 2.0 cc. total. Because of these findings, we

examined the possibility that in the centrifuged extract also the affinity of ATP might be lessened more for fructose than for glucose with decreasing concentrations of ATP. Direct measurements of transphosphorylation with very small amounts of ATP (15  $\gamma$  7-min. P and less) proved too inaccurate. However, the manometric and chemical determination of lactic acid formation under such circumstances did not bear out this hypothesis. These results with additions of small amounts of ATP (0–10  $\gamma$  7-min. P/cc.) were somewhat scattered. In the manometric setup, where 20–30 minutes elapse from the moment of warming up the enzymatic mixture to 30°C. until the coenzymes are tipped in, the addition of 5–10  $\gamma$  7-min. P ATP elicits only a very low glycolysis,  $Q_{La} < 5$ , often hardly recognizable at all. In the absence of added ATP no  $CO_2$  is evolved over that of the blank. But when the incubation starts at the moment where the enzymatic extract, kept at 0°C. is added to the prewarmed glycolytic mixture, as in the chemical experiments, in some cases appreciable amounts of lactic acid are formed from sugars with such small amounts of added ATP, either because in such a fresh extract the phosphorylating enzymes are much more active, or because the enzyme extract still contains preformed ATP. However, in spite of these differences, no systematic trend is observed, indicating that fructose reacts more slowly than glucose.

The greatly lowered glycolytic rate of homogenates compared with extracts may well be partly explained by the lowered concentration of ATP because of the structurally bound apyrase. But the difference of the turnover rate of fructose and glucose is an independent fact peculiar to the structural elements. In the homogenate, the hexokinase adsorbed on the particles is mainly responsible for the reaction of the sugars, because the concentration of ATP is usually too low for the dissolved hexokinase to be active. By the adsorption the affinities of the hexokinase for ATP and different sugars are probably greatly altered. The living cell presumably contains only adsorbed hexokinase. The ATP concentration in brain tissue of rats, according to Stone (27), amounts to 180  $\gamma$  7-min. P/g., and the concentration of cozymase, according to Axelrod and Elvehjem (28), to 350  $\gamma$  DPN/g. These concentrations are as high as the largest amounts added/cc. in our experiments. On the other hand, we do not know whether these "average" concentrations, determined by analysis of the whole tissue, are identical with the reacting concentrations, because part of the coenzymes will be protected by combination with inert protein or by

structural separation in the living cell from free access to the sugar. It is quite conceivable that the acting concentrations are as low as in homogenates where the difference between glucose and fructose is most marked.

## 6. FORMATION OF PHOSPHOGLYCERIC ACID FROM VARIOUS SUGARS

The picture gained from the preceding results is corroborated by the study of the oxidation-reduction step the components of which are pyruvic acid and hexose, while the chain of consecutive reactions is interrupted by NaF. In this case the added pyruvic acid is reduced to lactic acid while hexose is phosphorylated to hexosediphosphate and oxidized by way of triosephosphate to 3-phosphoglyceric acid. After attaining the equilibrium distribution with 2-phosphoglyceric acid, the reaction is stopped by NaF (29). In the presence of bicarbonate the reaction can be measured manometrically, since two moles of acid are formed from one mole of hexose as in normal glycolysis. The addition of NaF, in addition to canceling out the steps between phosphoglyceric and pyruvic acids, has the advantage of partly checking the apyrase. This delays the removal of ATP. The most clear-cut results are obtained with homogenate without addition of ATP. The traces contained in the tissue protected by NaF are needed for only one transphosphorylation/mole of triosephosphate. They are sufficient to allow a slow continuous turnover with little decline for more than an hour, provided that pyruvate, cozymase, and sugar are added in optimal amounts.

Two such experiments are presented in Figs. 11 and 12. They give an indication of the extremes encountered under similar conditions.

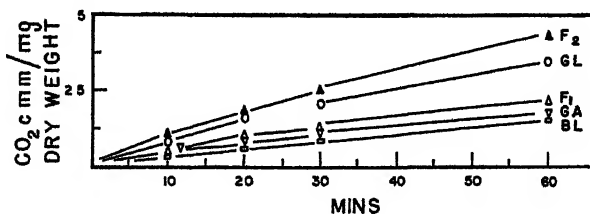


FIG. 11. Formation of Phosphoglyceric Acid in Presence of Pyruvate and NaF. Homogenate not frozen, no nicotinamide. 1.0 cc. homogenate (1:5) in total vol. of 1.6 cc. (No. 214). 0.1 cc. pyruvate *N*/10. 0.1 cc. NaF *N*/5. 0.3 mg. cozymase tipped in at start. □ Bl. blank. ○ Gl. 2 and 10 mg. glucose (identical curves). △ F<sub>1</sub> 2 mg. fructose. ▲ F<sub>2</sub> 10 mg. fructose. ▽ Ga 2 and 10 mg. galactose.

In Fig. 11 (No. 214) 0.2% and 1% glucose give, as usual, nearly identical values, not to be distinguished in the graph:  $Q_{CO_2} = 3.55$  and 3.9. Two-tenths *per cent* fructose and 1% fructose give quite different values,  $Q_{CO_2} = 2.1$  and 4.15, the former only one-third more than the blank. Galactose in 0.2% and 1% concentrations, gives a value which is a trifle higher than that of the blank. In Fig. 12 the rate of reaction of 1% fructose dropped after 20 minutes and became as low as 0.2% fructose.

But in the case where ATP (30  $\gamma$  of 7-min. P) and inorganic phosphate are added, the course is different for the first 20 or 30 minutes. Inorganic phosphate is now needed for completing the reaction since, from the two phosphate groups taken up for every triose group only the acylphosphate is transphosphorylated *via* ATP to hexose, and the

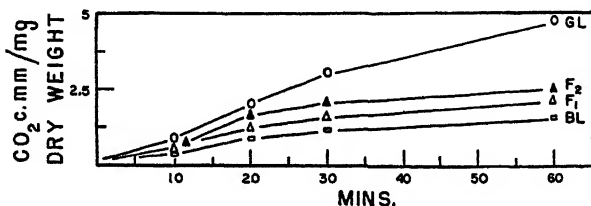


FIG. 12. Formation of Phosphoglyceric Acid in Homogenate (No. 216). 0.8 cc. homogenate (1:5) in total vol. 1.4 cc. 0.1 cc. *N*/10 pyruvate 0.1 cc. NaF. *N*/5 0.3 mg. cozymase tipped at start.  $\square$  BL. Blank.  $\circ$  GL. 3 mg. glucose.  $\triangle$  F<sub>1</sub> 3 mg. fructose.  $\blacktriangle$  F<sub>2</sub> 15 mg. fructose.

stable phosphate remains in the phosphoglyceric acid. A rapid reaction occurs, which, during 20 minutes, shows no difference in speed with glucose, and low and high concentrations of fructose. The acid formation of the blank, which is mostly decomposition of ATP, should be subtracted. In Table VIII the uncorrected as well as the corrected values are recorded and from the corrected values the  $CO_2$ /mg. of homogenate dry weight are calculated, allowing for a retention factor of 1.2 in the presence of phosphate. If the reaction were to continue with the same speed,  $Q_{CO_2}$  values of 12–15 would be obtained in one hour, about three times as much as in the absence of ATP. The same result was obtained by chemical determination of the lactic acid formed by reduction of the added pyruvate (see Table IX).

The homogenate again can be separated into the extract and the particles and the latter washed with Ringer solution. Both systems are

TABLE VIII

*Formation of Phosphoglyceric Acid with Added ATP and Hexoses*0.5 cc. homogenate (1:5) in 1.2 cc. total vol., 0.3 mg. cozymase, ATP with 30  $\gamma$  7-min. P,  $7.5 \times 10^{-3}$  M phosphate,  $1.5 \times 10^{-2}$  M NaF

No.	Pyruvate	Sugar	Mg.	Time	CO <sub>2</sub>	CO <sub>2</sub> cor- rected for blank	CO <sub>2</sub> /mg. dry weight (cor- rected for retention)
	<i>M</i>			<i>min.</i>	<i>mm.<sup>3</sup></i>	<i>mm.<sup>3</sup></i>	<i>mm.<sup>3</sup></i>
211	$5 \times 10^{-3}$			2	8.2		
				12	17.3		
				22	22.4		
		Glucose	4	2	16.6	8.4	.57
		Glucose	4	12	58.5	41.2	2.9
		Glucose	4	22	71	58.6	3.8
		Fructose	4	2	16.3	8.1	.55
		Fructose	4	12	69.7	52.4	3.6
		Fructose	4	22	82.5	60.1	4.4
		Fructose	20	2	22.9	14.7	1.0
		Fructose	20	12	88.5	71.2	4.9
		Fructose	20	22	105	82.6	5.6
213	$12 \times 10^{-3}$			22	45.6		
		Glucose	4	22	102	56.4	3.8
		Fructose	4	22	89.6	41.0	3.0
		Fructose	20	22	94.5	48.9	3.3

TABLE IX

*Reduction of Pyruvic to Lactic Acid with Formation of Phosphoglyceric Acid*  
*Chemical Determination of Lactic Acid*0.8 cc. homogenate (1:5) = 28 mg. dry weight, in 1.8 cc. total volume. 0.6 mg. cozymase, ATP with 75  $\gamma$  7-min. P,  $6 \times 10^{-3}$  M phosphate,  $4 \times 10^{-2}$  M NaF.  $8 \times 10^{-3}$  M pyruvate.

Sugar	Mg.	Time	Lactic acid formed	L. A. formation corresponds to	
				mm. <sup>3</sup> CO <sub>2</sub> / mg. d.w.	Q <sub>La</sub>
—	—	<i>min.</i>	$\gamma$		
		20'	25	0.2	—
Glucose	4	20'	536	4.8	14.5
Glucose	20	20'	651	5.8	17.5
Fructose	4	20'	486	4.3	13.
Fructose	20	20'	586	5.25	15.8
Galactose	4	20'	171	1.5	4.6

inactive without addition of ATP and cozymase. When these are added, the extract behaves like the unpoisoned extract, although giving a reduced rate as compared with the unchecked glycolysis. Such an experiment is reproduced in Fig. 13. Two-tenths and 1% glucose and 0.2% fructose give the same speed. One *per cent* fructose, not shown in the figure, gives a little less. Galactose shows only a slight reaction.

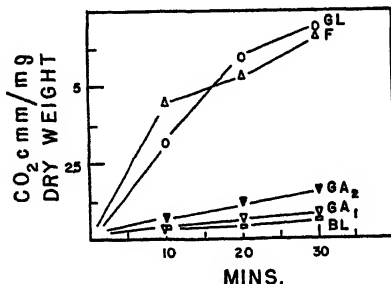


FIG. 13. Formation of Phosphoglyceric Acid in Extract (No. 222). 0.5 cc. extract (1:5) in 1.6 cc. total vol. 0.1 cc. *N*/10 pyruvate. 0.1 cc. *N*/2 NaF. 0.1 cc. *M*/10 phosphate. 0.05 coz. (0.3 mg.) + 0.05 ATP (20  $\gamma$  7-min. P) + 0.05 cc. bicarbonate tipped in 2 minutes before start.  $\square$  BL. blank.  $\circ$  GL. 3 mg. glucose.  $\triangle$  F 3 mg. and 15 mg. fructose (identical curves).  $\nabla$  GA<sub>1</sub> 3 mg. galactose.  $\blacktriangledown$  GA<sub>2</sub> 15 mg. galactose.

## DISCUSSION

Since the glycolytic cycle in mammalian tissue consists of about 12 consecutive steps and is dependent on the activity of an equal number of intermediary enzymes, several organic coenzymes, activators, and specific ions, it is clear that the rate of glycolysis in disintegrated tissue varies according to many circumstances. Nearly any desired rate of turnover can be obtained with the different techniques, through addition of suitable activators and varied concentrations of coenzymes.

The enzymatic extract of brain clarified by centrifugation is a relatively stable system. The results are fairly reproducible.  $Q_{La}$  values of 40–60, defined as above, are easily obtained with glucose as well as fructose, and remain constant for 30–60 minutes. This turnover rate, which signifies a 5- to 8-fold increase over that of tissue slices of grey-matter, stems from the high concentration of the added coenzymes, probably much higher than the true reacting concentration in the tissue, and from the partial removal of those enzymes, nucleotidase and apyrase, which destroy the coenzymes.

The homogenate, consisting of the dissolved enzymes plus the solid cell structures, is a much more complex system, giving less uniform results. In the extract just enough apyrase is left for maintaining the glycolytic turnover at a fairly constant rate. But so much apyrase is adsorbed on the particles that the added ATP is almost completely split in 1-2 minutes with the dilutions used here. Only by adding that same amount of ATP every two minutes which, in extracts, gives maximal turnover for 40 minutes with one addition, is it possible to maintain in a homogenate a glycolytic rate which approaches that of the extract. It is true that a similar result can be obtained by destruction of the interfering enzymes by means of distilled water. *The main problem, however, as to which factors condition the rate in the living tissue, cannot be answered in this way.*

The peculiar difference between the glycolysis of glucose and fructose, a characteristic feature of the sugar metabolism of brain, is absent in the extract and also in the distilled water homogenate, but is present in homogenates made with isotonic solutions. Here, in contrast to tissue slices, the components of the glycolytic system, sugars and intermediaries, are diffusible. One must infer that at low ATP concentrations the hexokinase adsorbed on the structural elements has a different affinity for the different sugars in distinction to the dissolved hexokinase. The reason for this difference remains unknown.

We are aware that additional factors may play some role. The explanation of Boyland that fructose penetrates brain and tumor cells more slowly than glucose, although inapplicable to brain homogenate, may be true for the whole tissue (8, 9). Moreover, factors of hormonal nature may influence the phosphorylation of glucose and fructose in a different way. That the hexokinase and the apyrase are the enzymes mainly responsible for the normal turnover rate of sugar in the living tissue is clearly evident from recent discoveries. Price, Cori and Colowick found that the hexokinase of rat tissues is inhibited by a hormone of the anterior pituitary lobe (k-fraction) (30), as well as by adrenal cortical hormone (31), and that these inhibitors are checked by insulin. These inhibitions were measured in the system of muscle or liver extract with ATP and glucose. We considered the possibility that a similar hormonal control would influence fructose differently from glucose or that the affinity of different concentrations of ATP to the sugars would be shifted to another range of ATP concentrations. Owing to the instability of the pituitary hormone, we could not prove



this point. No influence of cortical hormone with or without insulin could be seen on brain extracts of alloxan diabetic rats.<sup>6</sup> This possibility, however, is not ruled out.

On the other hand, the role of apyrase on the rate of fermentation in extracts has been brought out in experiments on yeast preparations (31). Hexokinase and apyrase, as well as the active concentration of the components of the adenylic system, seem to be the most important controlling factors in the glycolysis of the resting tissue. The concentrations of the other enzymes of the glycolytic cycle are probably not the decisive factor for the resting rate, although they may be called upon for functional activity.

The slow reactivity of galactose points to a different explanation. Galactose is probably transformed into one of the fermentable hexoses by an unstable factor which is mostly bound to the structural elements. Therefore, homogenates react somewhat better with galactose than extracts. This is brought out also in the experiments on transphosphorylation, and by the slight protection which galactose offers against the dephosphorylation of ATP.

#### ACKNOWLEDGMENT

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#### SUMMARY

1. Both tumor tissue and brain tissue glycolyze glucose much faster than fructose at low concentrations of the sugars, but enzyme extracts of these tissues, fortified with various coenzyme factors, glycolyze fructose more rapidly. Moreover, the  $Q_{La}$  for glucose is at least five times higher for extracts than for slices or homogenates of the tissues. These facts, previously known from the work of several authors, were confirmed and their underlying causes were investigated.

2. "Fortified" homogenate from brain, made with Ringer solution, shows nearly the same activity toward sugars as do slices. However, when the particles are removed by centrifugation, the resulting extract gives a high glycolytic rate ( $Q_{La}$  about 50), with fructose being

<sup>6</sup> We thank Dr. E. C. Kendall, Mayo Clinic, Rochester, Minn., and Dr. Inglo, Upjohn Company, Kalamazoo, Mich., for the supply of cortical extract, Dr. Lukens, Medical Research Inst., Univ. Penna., for frozen pituitary glands and the Eli Lilly Co., Indianapolis, Ind., for the supply of pure zinc-free insulin.

glycolyzed somewhat more quickly than glucose, and with galactose hardly being affected at all. The particles lose their inhibiting influence in distilled water.

3. One factor responsible for these differences is the concentration of ATP. Cozymase in the presence of nicotinamide is stable enough to insure maximal activity during 40–50 minutes, but the adenylypyrophosphatase (“apyrase”) bound to the particles is so active that the concentration of ATP decreases much more rapidly in homogenates than in centrifuged extracts. If enough ATP is added at short intervals (2 min.) during the incubation of the homogenate, and in this way a fairly high concentration is maintained, not only is the  $Q_{La}$  for glucose increased to 20, but about the same value is obtained for fructose.

4. The amount of transphosphorylation from ATP to hexose is the same for homogenates and extracts and for glucose and fructose at high and moderate concentrations of ATP.

5. Direct measurement of transphosphorylation from ATP to hexose is not feasible with very low concentrations of ATP (below  $15 \gamma$  7-min. P/cc.) but when tested by lactic acid formation, extract and homogenate behave differently under these circumstances. While with low ATP a marked difference between glucose and fructose appears in the homogenate, no such difference exists in extracts, even when, as in manometric experiments, the mean rate of glycolysis is very much lowered by a combined effect of a long preliminary warming up period with suboptimal doses of ATP.

6. The extent of oxido-reduction in the presence of pyruvate, NaF, and various hexoses shows a variation similar to that of lactic acid formation except that, because of the inhibition of apyrase by NaF, fructose reacts even at moderate concentrations of ATP as rapidly as glucose in homogenates, although not at very low concentrations (without addition of ATP over the preformed amount).

7. The question is discussed as to whether the reactivities of the different sugars can be explained on the basis of the ATP concentration.

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## Direct Estimation of Leucine Application to Milk and Certain Other Foodstuffs

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Rockland and Dunn (1) have recently shown that *Tetrahymena geleii* H may be used to determine tryptophan in unhydrolyzed protein. In our laboratory we have done considerable work on the analysis of protein for amino acids, without a preliminary hydrolysis, by use of mutants of *Neurospora crassa*. In this paper we will present some of the results of our investigations, since they may be of interest to other investigators and may stimulate further research on the direct microbiological assay of both pure proteins and foods for amino acids.

It has previously been pointed out by Block and Bolling (2, 3) and by Ryan and Brand (4), that the chemical methods for the estimation of leucine have not been entirely satisfactory. They are difficult to carry out, often require special equipment and, in many cases, do not give results in which the analyst can place confidence. In general, the chemical methods for amino acids require that the protein fraction of a food be purified and hydrolyzed or that suitable purification steps be taken after hydrolysis. The microbiological methods which utilize bacteria as the test organism present a considerable advance in amino acid analysis since they, in a large part, eliminate the purification steps. Kuiken *et al.* (5), Schweigert *et al.* (6), and Stokes *et al.* (7) have published microbiological methods for the determination of leucine. Snell (8) has presented a review on the use of bacteria for the determination of amino acids.

Rognery (9) has suggested that it may be possible to make direct assays for leucine without hydrolysis with the "leucineless" mutant 33757 of *Neurospora crassa* and has presented limited data supporting his suggestion. When leucine alone is to be determined the use of a direct method such as that suggested by Rognery offers a saving of time and material, since the purification and hydrolysis steps are eliminated and *Neurospora* require only a very simple medium. In contrast to the bacterial methods which require several vitamins and, with a few exceptions, a rather complete list of pure amino acids, the "leucineless" *Neurospora crassa* requires only biotin and leucine. If a food or protein is being analyzed for several amino acids, these advantages may be offset by the fact that the hydrolyzates must be prepared anyway and the results by bacterial microbiological methods may be slightly more suitable

for comparison with other published work, since the latter methods have already found widespread use.

We have used the "leucineless" mutant 33757 for the direct estimation of leucine in milk, other foods, and in a few samples of semi-purified proteins. The following double strength medium is used for the direct assays:  $(\text{NH}_4)_2\text{C}_4\text{H}_4\text{O}_6$  10 g.,  $\text{NH}_4\text{NO}_3$  2 g.,  $\text{KH}_2\text{PO}_4$  5 g.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1 g.,  $\text{CaCl}_2$  0.2 g.,  $\text{NaCl}$  0.2 g., sucrose 40 g., leucine 20 mg., biotin 16  $\gamma$ , water 1 l. and 10 ml. of a trace mineral mixture. The trace mineral mixture contains in mg./l.:  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  35,  $\text{H}_2\text{MoO}_4$  4,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  50,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  6,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  6,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  840. The medium is prepared fresh each day immediately before use. For the assays a suitable portion of a standard leucine solution or an unknown solution or suspension are pipetted into a 50 ml. Erlenmeyer flask, diluted to 5 ml. with water and 5 ml. of the double strength medium added. The standard series of flasks are prepared in duplicate and contain 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 mg. of *l*-leucine per flask. Homogeneous powdered foods or proteins may be weighed directly into the Erlenmeyer flask and covered with 10 ml. of dilute or single strength medium. The flasks are then plugged with cotton and sterilized.

Stock cultures of the "leucineless" mutant are maintained as suggested by Ryan and Brand (4). A large loopful of the conidia are transferred aseptically to 10 ml. of sterile distilled water and a spore suspension formed by shaking until uniformly turbid. One drop of the spore suspension is used to inoculate the medium in each flask as soon as the contents of the flask are cool after sterilization. When unhydrolyzed proteins are used as a source of leucine for the "leucineless" *Neurospora crassa* it is necessary that some proteolytic enzyme be present. If a small amount of leucine is present in the basal medium, mycelium growth is initiated from the spores and proteolytic enzyme is produced. If the inoculum is a direct transfer of actively growing mycelium, already containing some proteolytic enzyme, the addition of leucine to the basal medium is unnecessary. We have found the use of a spore suspension more convenient than the use of mycelium transfers.

An incubation period of 8 days at 25–30°C. has been used for the mold growth. This permits approximately maximum growth. The flasks are shaken gently twice daily. This step may be omitted but it facilitates harvesting, and may aid the digestion of solid samples which cling to the sides of the flasks. At the end of the incubation period the flasks are steamed for 5 minutes, the mycelium harvested while still warm, pressed dry between layers of paper toweling or filter paper, dried at 100°C. for at least 3 hours and weighed. This is essentially the method of harvesting *Neurospora* mycelium recommended by Stokes *et al.* (10).

Two samples of leucine obtained from different sources were used as standards. They gave identical *Neurospora* growth. One of the samples was shown by the manufacturer's analyses to be highly purified.

The samples used in our investigation were all commercial products, with the exception that one sample of Casein (No. 5) was prepared in the laboratory from fresh skim milk according to Cohn's method as given by Schmidt (11).

For comparative purposes we have analyzed the samples for leucine by the use of the "leucineless" *Neurospora crassa* and with the *S.*

*faecalis* method of Stokes *et al.* (7). In the latter method a 1 g. sample is hydrolyzed with 10% HCl for 10 hours. In Table I the results by the two methods when applied to foodstuffs are compared. While the

TABLE I  
*Comparison of Leucine Results Secured with "Leucineless" Neurospora crassa and S. faecalis on Foodstuffs*

Food	Per cent nitrogen	Per cent leucine of the food		"Leucineless" result expressed as per cent of <i>S. faecalis</i> result
		"Leucineless" <i>Neurospora</i> method	<i>S. faecalis</i> method	
Dry skim milk 1	5.80	4.10	3.67	112
Dry skim milk 2	5.78	4.04	3.79	106
Dry skim milk 3	5.49	3.83	3.72	103
Dry skim milk 4	5.44	3.73	3.88	96
Dry skim milk 5	5.75	4.05	4.11	99
Dry skim milk 6	5.56	4.30	4.08	105
Dry skim milk average	5.64	4.01	3.87	103
Dry whole milk 7	3.97	2.91	3.02	96
Dry whole milk 8	3.94	2.96	3.07	96
Dry whole milk 9	4.03	3.21	3.04	106
Dry whole milk 10	3.86	3.10	2.63	118
Dry whole milk 11	3.84	3.15	2.97	106
Dry whole milk 12	4.02	3.16	3.09	102
Dry whole milk average	3.94	3.08	2.97	104
Dried brewer's yeast	8.90	3.37	3.74	90
Corn germ (defatted)	2.86	1.20	1.25	96
Wheat germ (defatted)	5.92	1.85	2.17	85
Soybean flour	7.91	3.67	3.80	97
Potato flour	1.13	0.37	0.39	95
Egg albumin (Commercial dried egg white)	12.21	7.80	6.40	122

results by the two methods are not identical, fair to good agreement is shown in most cases and neither method consistently gives higher or lower results. In Table II a comparison is made of the results given

TABLE II  
*Leucine Content of Semi-Purified<sup>1</sup> Proteins*

	Per cent nitrogen	Per cent ash	Per cent leucine <sup>2</sup>		"Leucineless" result expressed as per cent of <i>S. faecalis</i> result
			"Leucineless" <i>Neurospora</i> method	<i>S. faecalis</i> method	
Casein No. 2 (vitamin free)	15.2	1.32	10.5	9.7	108
Casein No. 3 (vitamin free)	15.8	1.44	11.1	9.8	113
Casein No. 4 (acid washed)	14.9	2.78	9.5	9.3	102
Casein No. 5 (reprecipitated)	16.1	1.57	11.2	10.1	111
Gelatin No. 1	17.8	0.42	2.3	2.7	85
Gelatin No. 2	17.7	1.26	2.7	3.3	82
Crystalline bovine plasma albumin (powder dried from frozen state)	15.7	0.22	9.2	10.5	88
Fraction I from bovine plasma (fibrinogen)	11.2	9.88	5.5	4.9	112
Fraction V from bovine plasma	16.5	0.96	10.1	10.3	98

<sup>1</sup> The proteins are designated as semi-purified, since, with one exception, they were commercial samples, analyzed as purchased, without further purification or tests for purity in our laboratory. Casein No. 5 was prepared in our laboratory. The plasma proteins were purchased from the Armour Laboratories.

<sup>2</sup> The data are on a moisture free basis but are not corrected for ash.

by the two methods on semi-purified proteins. The results show about the same agreement as those on foodstuffs. For results secured by other investigators, cf. Refs. 2, 3, 4, 5, 6 and 7. The values obtained by a chemical method and reported by Block and Bolling (2, 3) are somewhat higher, and those obtained by other microbiological methods slightly lower, for casein and milk than the values we have found by use of the direct *Neurospora* method.

We have made only limited application of the direct method for leucine to protein-rich materials other than milk. Although the results are quite promising, further tests are necessary to establish whether or not certain proteins may be resistant to *Neurospora* proteolytic enzymes. The early growth with gelatin and egg white is slow and the final result with gelatin may be too low.

It is obvious that foods high in fiber or other substances which would be mechanically bound in the mycelium cannot be assayed by the direct method as outlined above.

The response of "leucineless" to leucine or to leucine combined in protein is quite uniform. Standard curves made at different times are very similar, duplicates agree well and results on different samples of the same material fall in a narrow range when compared on a protein basis. We have not experienced the trouble with adaptations mentioned by Ryan and Brand (4). Complete adaptations rarely occurred and partial adaptations were suspected in only a few instances. In general, only a few values at the extreme ends of the assay curve were excluded from the averages.

The simplicity of the direct *Neurospora* method for leucine makes it of special interest in exploratory work.

The "arginineless" mutant No. 36703 of *Neurospora crassa* (12) has been used for direct arginine assays on milk, casein, and other foods. Only moderate success was experienced with the direct arginine assays, and the results will not be reported in detail here. Apparently the "arginineless" mutant No. 36703 is easily inhibited by materials which may occur in foodstuffs. When this mutant is applied to the direct estimation of arginine in foods, there is occasionally poor agreement between duplicates and a tendency for results to drift downward with increasing arginine levels. When precise results are required, chemical methods and other microbiological methods appear to be preferable to the direct "arginineless" method we have used. However, the simplicity of the direct method may make it of value in some comparative and exploratory work. It should not be inferred from the present investigation that a *Neurospora* method of high precision for arginine could not be developed either through the use of a more refined technique or through use of another mutant. This investigation has been limited to one of many possibilities.

#### ACKNOWLEDGMENTS

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## SUMMARY

1. A direct *Neurospora* method for leucine in foodstuffs has been presented. This method does not require a preliminary hydrolysis of the protein in the foodstuff before assaying.

2. The results secured by the direct *Neurospora* method are compared with those secured by another microbiological method which includes acid hydrolysis and final assay with *S. faecalis*.

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# Oxidation-Reduction Potential of Cytochrome c

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## INTRODUCTION

Several determinations of the oxidation-reduction potential of cytochrome c have been reported. Table I presents a review of these.

Of these investigators, only Green found a variation in the slope of the  $E'_0$ -pH curve, while the others found a constant potential between pH 5 and 8, as far as they extended their investigations to pH values other than 7. Green's results, however, were obtained by reduction with sodium hydrosulphite, which, according to several authors, will give false potentials (8, 9). We had the same experience. At pH 7, for example, we found the  $E'_0$  value to be +0.124 volts if reduction was performed with sodium hydrosulphite, which is the same value as that reported by Green. However, the conditions are somewhat vague, and there are several factors to be kept in mind. Green worked with yeast cytochrome while the others used animal heart cytochrome, excepting Coolidge, who obtained +0.260 volts for yeast cytochrome

TABLE I

*Earlier Determinations of the Oxidation-Reduction Potential of Cytochrome c*  
rH 18.3-19.9 corresponds to +0.120-+0.180 volts

Investigator	pH	$E'_0$ at pH 7 in Volts	Reference
Coolidge	7	+0.260	1
Stone and Coulter	7	+0.280	2
Stotz, Sidwell & Hogness	5-8	+0.262	3
Wurmser & Filitti-Wurmser	5-8	+0.253	4
de Tocuf	7	rH 18.3-19.9	5
Ball	muscles <i>in situ</i>	+0.27	6
Green	4.6-9.2	+0.127	7

with very impure preparations and with sodium hydrosulfite as reductor.

The narrow limits of the pH region investigated seems to depend upon the authors' desire to treat the problem from a physiological point of view. From the  $E'_0$ -pH curve one can, however, get some information on the heme-linked groups in the enzyme. As was shown by Clark *et al.* (10), a decrease of  $dE'_0/dpH$  represents the manifestation of a dissociation in the oxidant, and an increase represents a dissociation in the corresponding reductant. As cytochrome c has a very broad range of pH stability, it may be supposed that several dissociation constants for heme-linked groups could be detected or confirmed if the oxidation-reduction potential was determined over the whole pH scale. This paper, dealing with pH 4-8, and giving some preliminary values from pH 1 to 11.1, is the first report on these investigations.

## EXPERIMENTAL

### *Preparations*

Cytochrome c was prepared according to the method of Theorell and Åkesson (11). It has an iron content of 0.43% and proved to be pure when tested in the Tiselius electrophoresis apparatus (12).

The dyc-stuffs used were of the quality "Schering-Kahlbaum *pro analyse*." Their further purification was not undertaken.

Reducton, prepared according to the method of Euler and Martius (13) and recrystallized from hot water, was used as reducing agent. This is a very convenient reductor, as it is not oxidized by atmospheric oxygen, but reduces cytochrome immediately in neutral and alkaline media and has a redox potential sufficiently low to avoid interference with the cytochrome potential (14). It was added either in solution from a burette or in the solid state. In the latter case, the gas outlet tube was uncovered for a moment and a few grains of reducton were placed in the solution.

Sometimes reduction was performed with hydrogen and platinized platinum or at pH 1.02 with titanous chloride in 0.1 *N* hydrochloric acid.

### *Apparatus*

"Filter cuvettes" (50 × 90 mm., optical depth 14.28 or 10.00 mm.) with plexiglas covers and sealed with low melting paraffine, were used as reaction vessels. In the covers were fixed two or three bright platinum electrodes, a salt bridge (3% agar in saturated potassium chloride solution) to a saturated calomel electrode, gas inlet and outlet tubes and burettes. An electron tube potentiometer (Radiometer, Copenhagen, type PHM 3f) was used for registering the potentials. Continuous stirring was

obtained with an iron cylinder, covered with a plexiglas cylinder (outer diameter 9 mm., length 12 mm.), which was kept rolling by means of a permalloy magnet.

All measurements were performed in a nitrogen atmosphere. Commercial nitrogen was purified by passage through a glass tube filled with copper wire and heated to 500°C., and then washing with alkaline pyrogallol solution.

The cuvette was placed in a special holder about 2 cm. from the exit slit of a Warburg-Negelcin spectrophotometer (15) at right angles to the light beam. On the same holder was fixed a reference cuvette with pure water. The holder was movable so that either the cuvette with test solution or the reference cuvette could enter the light path. With this arrangement it was possible to read the redox potentials and pH and determine spectrophotometrically the degree of oxidation without opening the system.

No liquid thermostat was used. The temperature of the air in the room was kept constantly at  $+20^\circ \pm 0.5^\circ\text{C}$ .

From the recorded potentials ( $E_A$ ), the potentials at 50% oxidation at the same pII ( $E'_0$ ) were calculated according to the familiar formula

$$E_A = E'_0 + \frac{RT}{nF} \log_e \frac{[\text{oxidant}]}{[\text{reductant}]} \quad (1)$$

The ratio  $[\text{oxidant}]/[\text{reductant}]$  was calculated in the following way. The extinctions of completely oxidized and reduced cytochrome c solutions of about the same concentrations as those used in the redox experiments were measured at two suitable wavelengths  $\lambda_1$ , and  $\lambda_2$ . From these four numbers the ratios  $\epsilon_{\lambda_1}/\epsilon_{\lambda_2}$  were calculated for ferri- and ferrocytochrome c and also, by straight line interpolation, for their mixtures ( $\epsilon = \log_{10} I_0/I$ , where  $I_0$  = intensity of incident light and  $I$  = intensity of transmitted light). Thus curves ( $\epsilon_{\lambda_1}/\epsilon_{\lambda_2}$  plotted against *per cent* oxidation) were prepared for integral pII values from 4 to 11, and from these a measured ( $\epsilon_{\lambda_1}/\epsilon_{\lambda_2}$ ) for the test solution in a redox experiment gave the degree of oxidation. If a measurement was performed at, for example, pH 7.60, the degrees of oxidation at pH 7 and 8 were read from the curves and the desired value interpolated from them. This method is not strictly correct, as the changes in the degree of oxidation from one pH to another for a given ratio do not follow a straight line but some sigmoid curve. The error, however, will stay far below 0.5% in the degree of oxidation, which is negligible between 25 and 75% oxidation of the cytochrome. As the cytochrome concentration was known, the degree of oxidation could, of course, be calculated from one single wavelength, but the method used prevents error due to evaporation by the nitrogen gas stream and other concentration changes. When a mediator was used, corrections for its light absorption had to be made. For calculating this, one must know the mediator's concentration, but the real changes of this are small, so changes in the mediator's light absorption due to evaporation of the solution are negligible when compared with the total light absorption.

The saturated calomel electrode was checked against a hydrogen gas electrode with pII 4.62 (standard acetate solution) and proved to give the potential  $+0.2486$  volts.

Except for the first few minutes, the platinum electrodes agreed with each other within  $\pm 0.001$  volts.

## RESULTS

*Spectrometric Measurements*

The two wavelengths used for neutral and alkaline solutions were 550  $m\mu$ , where ferrocytochrome *c* has an intensive absorption, and 565  $m\mu$ , where ferricytochrome *c* shows an absorption band. Fig. 1

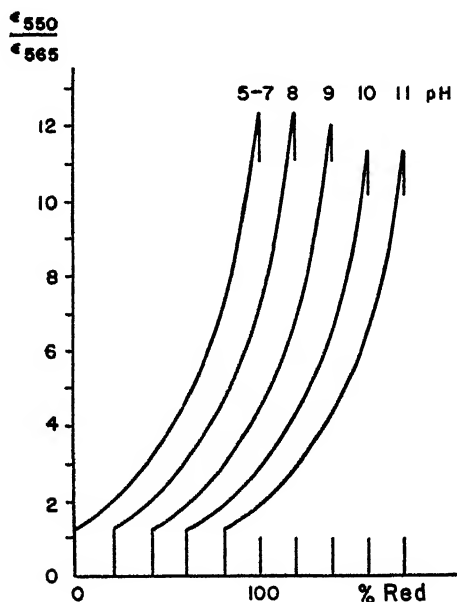


FIG. 1. Variations of  $\epsilon_{550}/\epsilon_{565}$  with *per cent* Oxidation. As the curves for different pH values have nearly the same shape, their starting points are shifted along the *x*-axis. Thus, for example, the curve at pH 9 begins below the point for "20% reduced" at pH 8. For use of the curves see text.

shows the ratios mentioned above for different degrees of oxidation and at different pH values. During these determinations it was found that the  $\epsilon_{550}$  value for ferrocytochrome *c* always became higher at alkaline than at neutral reactions, and that this change was reversible. It therefore seemed of interest to investigate whether there was an optically operable dissociation constant in ferrocytochrome *c* at slightly alkaline reaction. The technique described by Theorell and Paul (16) was used. From the figure one can see that there appears to be an in-

crease of about 4% in the light absorption when pH is raised from 6 to 11. The molar extinction coefficient  $E_{550}$  for ferrocytochrome c is  $6.52 \times 10^4$  at neutral reaction (17),  $E$  being defined as

$$E = \frac{1}{c} \times \frac{1}{d} \times \log_e \frac{I_0}{I}, \quad (2)$$

where  $c = M/l.$  of solution and  $d =$  optical depth in cm. The wave length  $550 \text{ m}\mu$  was used because a slight reoxidation would be easily detected there as a considerable decrease of the extinction.

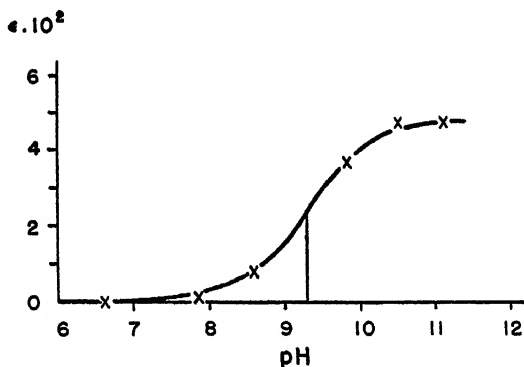


Fig. 2. Optical Determination of the Ferrocytochrome c Dissociation Constant at pH 9.28. 7.00 ml. of  $3.31 \times 10^{-4} \text{ M}$  cytochrome c solution was diluted to 100.0 ml. with  $M/15 \text{ KH}_2\text{PO}_4$  solution. From this solution 7.00 ml. were pipetted into each of two cuvettes with optical depth 1.996 cm. Fresh samples were taken for each measurement. 0.05–0.95 ml. of 1.0  $\text{M}$  KOH was added to one cuvette and the same volume of water to the other. The samples were reduced with about 10 mg. of dithionite. pH was determined with a glass electrode after the optical measurements. In the reference cuvette with cytochrome + water pH was 6.1–6.2. The values are corrected for the dilution.

The change follows a simple dissociation curve with  $\text{pK} = 9.28$ . Similar results were obtained with other preparations of the same purity. Thus ferrocytochrome c has an optically operable dissociation constant with  $\text{pK} 9.28$ .

#### *Oxidation-Reduction Measurements*

Owing to the difficulty of obtaining suitable mediators, some experiments were performed with cytochrome alone. In these cases, the

TABLE II

*Experiment on Redox Measurements*

A mixture of 1.10 ml. of  $9.70 \times 10^{-4}$  *M* cytochrome *c* + 27.00 ml. of *M*/30 phosphate buffer (pH 6.81) + 0.63 ml. of  $1 \times 10^{-4}$  *M* 2,6-dichlorophenolindophenol was partly reduced with platinum and hydrogen and then anaerobically pipetted into the cuvette. Reduction solution  $0.0014$  *M*.  $E_{550} = 2.35 \times 10^4$  and  $E_{545} = 2.76 \times 10^4$  for the mediator. Optical depth of the cuvette = 10.00 mm. pH determined with the glass electrode.

$E_h$	Readings		$E'_s$ for Med	Reduction of Mediator	[Mediator]	Corr for Med Light Abs		Corrected $\lambda$ values		$\frac{\epsilon_{545}}{\epsilon_{550}}$	Reduction of Cytochrome	$E'_s$ for Cytochrome	pH
	$\epsilon_{545}$	$\epsilon_{550}$				550 $m\mu$	565 $m\mu$	550 $m\mu$	565 $m\mu$				
soln			volts	per cent	$M \times 10^4$						per cent	volts	
+0.2647	0.942	0.367	+0.2320	7.0	2.19	0.021	0.024	0.921	0.343	2.69	33.0	+0.247	6.80
0.05 ml. of reduction solution added													
+0.2625	1.010	0.354	+0.2320	8.9	2.19	0.021	0.024	0.989	0.330	3.00	39.5	+0.252	6.80
0.10 ml. of reduction solution added													
+0.2374	1.172	0.282	+0.2320	39.5	2.18	0.013	0.016	1.159	0.266	4.36	59.0	+0.247	6.80
0.20 ml. of 1 <i>M</i> NaOH added													
+0.2071	1.207	0.285	+0.1750	7.3	2.17	0.021	0.024	1.186	0.261	4.54	60.5	+0.218	7.60
0.10 ml. of 1 <i>M</i> NaOH added													
+0.1750	1.215	0.286	+0.1440	7.9	2.16	0.020	0.024	1.195	0.262	4.56	60.0	+0.185	8.09

time necessary to reach stable potentials varied from 4 to 10 hours. Of the dyestuffs available, only 2,6-dichlorophenolindophenol had an  $E'_0$  value high enough to be suitable for cytochrome (18). With this mediator, the potentials reached equilibrium after about 30 minutes. A typical experiment is shown in Table II.

The number of electrons exchanged on oxidation of ferrocytochrome c was calculated from the  $E_h$  values at pH 6.80 (Fig. 3) and, in agreement with earlier investigations, found to be 1.

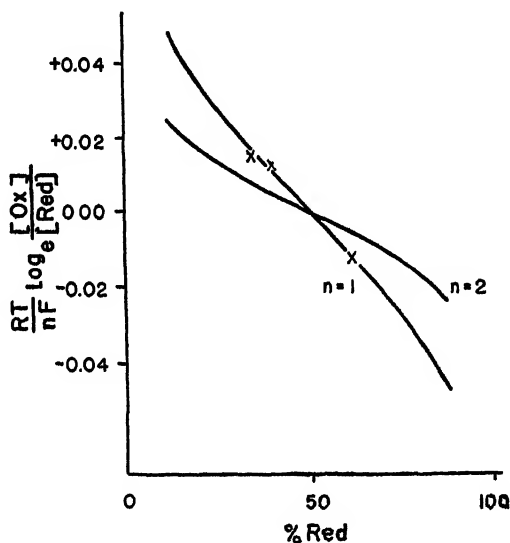


FIG. 3. The Values of the Last Term in (1), Obtained at pH 6.80, Plotted against per cent Reduction of Cytochrome. The solid lines represent the two possibilities  $n = 1$  or  $n = 2$ .

The results obtained are assembled in Fig. 4. From the figure one can see that a dissociation constant in ferricytochrome c appears at about pH 7. The constant was calculated according to the formula

$$E'_0 = E_m + \frac{R T}{n F} \times \log_e \frac{[H^+]}{[H^+] + K}, \quad (3)$$

where  $E_m$  is defined as

$$E_m = E_0 - \frac{R T}{n F} \times \log_e \frac{1}{K}, \quad (4)$$



and  $E_0 = E'_0$  at  $\text{pH} = 0$ .  $K$  was found to be  $1.38 \times 10^{-7}$  and thus  $\text{p}K = 6.86$ .

Some preliminary results at extreme acidity and alkalinity may be mentioned as certain conclusions can be drawn from them. At  $\text{pH}$  1.02 an equivalent mixture of cytochrome and methylene blue was reduced with titanous chloride, dissolved in 0.1  $N$   $\text{HCl}$ .  $E_h$  was determined potentiometrically as described above and colorimetrically as described by Stotz, Sidwell and Hogness (3). The  $E'_0$  values agreed within  $\pm 0.003$  volts with the mean value  $+0.357$  volts, when different oxidation degrees were used.

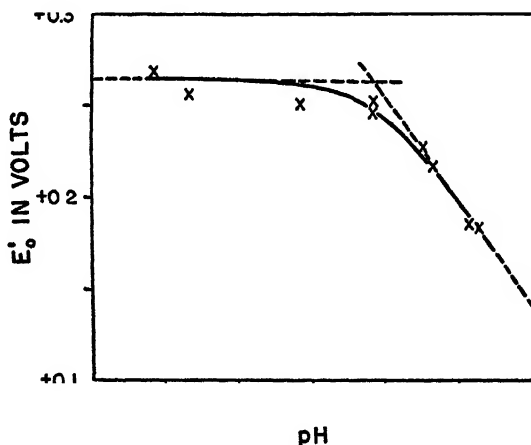


Fig. 4.  $E'_0$  Values between  $\text{pH}$  4 and 8. The solid line represents equation (3) with  $E_m = +0.266$  volts and  $K = 1.38 \times 10^{-7}$ . The slope between  $\text{pH}$  8 and 9 is  $-0.056$  volts/ $\text{pH}$ .

At alkaline reaction we found  $E'_0$  at  $\text{pH}$  8.98 to be  $+0.139$  volts; at  $\text{pH}$  9.73,  $+0.118$  volts; at  $\text{pH}$  10.70,  $+0.064$  volts; and at  $\text{pH}$  11.1,  $+0.032$  volts. These values were obtained potentiometrically, and no mediator was used.

#### DISCUSSION

The high  $E'_0$  value  $+0.357$  volts at  $\text{pH}$  1.02 indicates that, at least one, and possibly several, potentiometrically operable dissociation constant(s) must exist in ferrocytochrome c between  $\text{pH}$  1 and

4. From the investigations of Theorell and Åkeson (17) it is known that ferricytochrome c has two dissociation constants below pH 4. Now a dissociation constant in an oxidant is cancelled by a dissociation constant in the corresponding reductant if they differ by less than 0.2 pH units (19). One must, therefore, calculate with the possibility that two constants are potentiometrically inoperable.

The  $E'_0$  values at alkaline reactions indirectly confirm a ferrocytochrome dissociation constant at pH slightly above 9. Theorell and Åkeson determined spectrophotometrically a ferricytochrome constant at pII 9.35 (17). If no ferrocytochrome constant cancelled this, an  $E'_0$  value of about  $-0.038$  volts at pH 10.7 would be expected instead of the  $+0.064$  volts experimentally found.

The dissociation constant with pK 6.86 is not found sooner. Taylor and Hastings (8) found a dissociation constant in ferrihemoglobin with about the same value (6.65). Coryell and Pauling (19) compared this with the pK values for ionization of substituted imidazoles, which were predicted by them to be about 7. Thus, the new dissociation in ferricytochrome c falls within the region of dissociations in the imidazole nucleus, but why it was not found by acid-base differential titrations of ferri- and ferrocytochrome c (17) remains to be explained.

The redox measurements of cytochrome c are continued, and a general discussion of the problem will rest until complete experimental data are available.

### SUMMARY

On determinations of the oxidation-reduction potential of cytochrome c a potentiometrically operable dissociation constant in ferricytochrome c was found with pK = 6.86. Spectrophotometrically a dissociation constant in ferrocytochrome c was found at pH 9.28.

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## Book Reviews

**Advances in Enzymology, Volume VI.** Edited by F. F. NORD. Interscience Publishers, Inc. 1946. 563 pp. Price \$7.00.

The eleven articles which comprise the present volume deal with a wide variety of subjects; some of these are strictly in the enzyme field while others cover borderline areas. Most of the articles provide excellent and well-organized summaries of a circumscribed topic and as such are of great value. In many cases the interest is heightened not only by the intimate knowledge the author shows for his subject but also by the inclusion of unpublished material. A good subject and authors index increase the usefulness of this publication.

In order of appearance there is Gale's article on bacterial amino acid decarboxylases, giving remarkably complete information on the nature, action, formation, and distribution of this class of enzymes. Gale's own work in this field is well known; the separation of the enzymes from the bacterial cell and their purification led to the recognition that a specific coenzyme is part of the enzyme system. This coenzyme, through the work of several authors, has now been identified as pyridoxal phosphate. Another vitamin (pyridoxine and related compounds) has thus been shown to function as a necessary component of an enzyme system.

Sevag's article on enzyme problems in relation to chemotherapy contains over 200 references, but in several cases will not please the authors quoted. There is considerable inaccuracy and a tendency to fit diverse experimental data, on the basis of superficial examination, into a common pattern. The result is an article which contains valuable material but is not as clear and readable as might be desired. Woolley's article on biological antagonisms between structurally related compounds covers a similar field and starts with the basic observation of Woods on the antagonism between sulfanilamide and *p*-aminobenzoic acid in bacterial growth. A number of such pairs have since been investigated by Woolley and others among the vitamins, amino acids, purines and other biological systems. In many cases the structural analogues of vitamins (*e.g.* pyriithiamin) cause not only inhibition of bacterial growth (in those species which require the vitamin as a growth factor) but also produce typical deficiency diseases in animals. Woolley also discusses some general aspects of inhibition, the implication for pharmacology and the possible mechanism of inhibition, and warns against the premature adoption of working hypotheses.

Engelhardt has written a comprehensive review of the adenosinetriphosphatase activity of myosin which was originally discovered by him. Recent work of the Szent-Gyorgyi school is included, and the influence of adenosinetriphosphate on myosin threads and on the viscosity and flow birefringence of myosin solutions is discussed. On the basis of the observed rate of splitting of adenosinetriphosphate by myosin solutions it can be calculated that about 0.1 cal./g. muscle/sec. are liberated in this reaction, a value which is similar to that observed during tetanic contractions of muscle.

The article by the late Dr. C. L. Hoagland, whose premature death must be regarded as a great loss to science, deals with the altered metabolism in diseases of muscle. A discussion of the changes in the composition and metabolism of muscle following denervation or deprivation of vitamin E is included. The fact is emphasized that changes in the composition of muscle in progressive dystrophy are difficult to evaluate, because an adequate method for estimating the residual mass of contractile muscle fibers is not available. A rough estimate is obtained by measuring the urinary output of creatinine. A more accurate estimate can probably be derived from a determination of the myosin content of muscle.

Lipmann's article on acetyl phosphate deals with the metabolism of this compound in bacteria and its possible relation to photosynthesis. The model reaction is, acetyl-phosphate +  $H_2$  +  $CO_2 \rightleftharpoons$  pyruvate + phosphate. In animal tissues rapid destruction of acetyl phosphate by a heat-stable enzyme has so far obscured results. A study of the activation of acetate in such reactions as the acetylation of sulfanilamide or of choline has led to the discovery of a new coenzyme, presumably a nucleotide. A recalculation of the change in the free energy for the hydrolysis of phosphopyruvate yielded a value of 16 kg.-cal.

Microbial assimilation, particularly of carbon, is reviewed by Clifton, as well as the effect of poisons such as azide and dinitrophenol on the assimilatory process. Complete carbon balances are presented for a number of organisms. The data suggest that there is a stoichiometric rather than energetic relationship between the anabolic and catabolic activities of the cell.

Other articles which cannot be reviewed here in detail include chemical changes in harvested tobacco leaves (Frankenburg), the action of amylases (Hopkins), the amylases of wheat in relation to milling and baking technology (Geddes), and tocopherol interrelationships (Hickman and Harris).

CARL F. CORI, St. Louis, Missouri

**Chemotherapy, Advancing Fronts in Chemistry, Vol. II.** Edited by WENDELL H. POWERS, Reinhold Publishing Corp., New York, 1946. 156 pp. (illustrated). Price \$3.50.

This small volume is composed of six chapters based upon lectures presented at a Symposium on chemotherapy held in 1945 at Wayne University. A further lecture on antibiotics was presented by H. E. Carter but not prepared for publication. The chapters are not correlated with one another, but each includes a survey of the history and present status of the particular topic under discussion.

Chapter I, by W. F. Feldman on "Chemotherapy in Experimental Tuberculosis," includes an introductory section of considerable merit consisting of a critical discussion of the requirements of a satisfactory chemotherapeutic agent and of the experimental procedures best suited to the search for a satisfactory drug. Although the work surveyed did not lead to any tuberculotherapeutic substances of great promise, it is encouraging to note that definite progress was made and that Dr. Feldman does not despair of the possibility of developing a chemotherapy in tuberculosis.

In a chapter on "Synthetic Antiplasmodics," F. F. Blicke gives examples of the way in which palliative drugs have been discovered by the synthesis of models of parts of the structures of the two plant drugs atropine and papaverine. This topic is actually

outside the scope of "chemotherapy" as the term is ordinarily understood: the prevention or treatment of a disease (usually infectious) by a specific chemical agent.

An article by E. H. Northey on the "Chemistry of the Sulfa Drugs" contains interesting comments on the types and characteristics of those members of this gigantic collection of related synthetic compounds that have proved the most useful. Northey is of the opinion that the peak of pharmacological potency among sulfa drugs probably has already been realized and that future advances are more likely to be found in chemotherapeutic agents that act by a different mechanism.

A chapter by H. S. Mosher entitled "The Antimalarial Problem" is introduced by an informative and useful summary of the history of malarial therapy and of the urgent problem of malaria control in the past war. This is followed by a description of the antimalarial research conducted at Pennsylvania State College under the OSRD.

The diversified field of "Organometallic Compounds as Chemotherapeutic Agents" is surveyed in a well-documented article by C. K. Banks. Although many compounds of widely varying types have been applied to a number of different uses, a few generalizations are suggested; for example that the organic derivatives of gold, bismuth and silver are effective in therapy only to the extent that inorganic forms of the elements are effective, and any advantages are associated with factors of improved absorption or retention of the element itself.

The concluding chapter, entitled "Past Developments and Present Needs in the Chemotherapy of Parasitic Diseases" is by W. H. Wright, of the U. S. Public Health Service. The discussion in this chapter is of broad scope and touches some of the fields of the other chapters; particular attention is given to anthelmintics. I was impressed by Wright's honest and stimulating concluding comments: ". . . this discussion has dealt only with the more superficial phases of the subject. . . . the experimental work in this field to date has been superficial in character. . . . we have hardly touched on the broad underlying principles which will require elucidation before we can hope to achieve ultimate goals." The general impression gleaned from this collection of lectures amply bears out Dr. Wright's view. Chemotherapy at present seems to be an ill-defined collection of miscellaneous empirical observations concerning the overall effect of the action of various chemicals against various infections, and a pressing need exists for the development of basic principles concerning the nature of drug action.

LOUIS F. FIESER, Cambridge, Mass.

**The Chemistry of the Carbon Compounds. Volume III. The Aromatic Compounds,** by VON RICHTER AND ANSCHÜTZ. Newly translated from the twelfth German edition by A. E. MEE, Glasgow, Scotland. Elsevier Publishing Co., Inc., New York. xviii+794 pp. Price \$15.00.

*The Chemistry of the Carbon Compounds* by Richter-Anschütz is not a textbook of organic chemistry, but represents a reference work which may be termed a "pocket edition" of Beilstein. The treatise consists of four volumes which deal with the Aliphatic Series, the Alicyclic Series and Natural Products, the Aromatic Compounds, and the Heterocyclic Series and Free Radicals. The present volume is concerned with the chemistry of the aromatic compounds, and represents a translation from the

twelfth German edition (published in 1935). The book begins with a brief summary on the general properties of benzene compounds, their isomerism, the determination of the position of the substituents, isomerism of the polysubstitution products, substitution rules, and finally the structure of the benzene nucleus. These more general survey chapters lead to a discussion of the formation and rupture of the benzene ring and to a well organized compilation of the physical and chemical properties of a great number of benzene derivatives. This is followed by an analogous treatment of the polynuclear hydrocarbons and their derivatives. In contrast to the German edition, where the references are given to *Chemisches Centralblatt*, the present translation refers to the original literature and authors' names have been added.

With the exception of the chapter on the structure of the benzene nucleus, which has been rewritten and which summarizes in a concise manner the modern views on the subject, the reviewer was unable to detect notable differences from the original text. Obvious misprints—for example, the structure of indene on page 590, or the formula of the diazonium salt of *o*-amino- $\alpha$ -phenylcinnamic acid on page 669—have been noncritically copied from the original. In addition, the book contains a number of errors, such as the misprints of the benzene structures on pages 28, 29, 30, and 32, and the mix-up in *para*- and *meta*-xylene on page 42.

The publishers' decision to publish an exact translation of a German text which appeared in the year 1935 seems unfortunate in view of the considerable progress, both theoretical and practical, which has since been made in the field of aromatic chemistry.

Richter's treatise represents a valuable addition to the library of every organic chemist, as it contains a tremendous wealth of information on organic chemistry. However, it is to be hoped that it will be possible in the near future to revise the present edition and bring it up to date.

Wartime restrictions have interfered with the printing of the volume, and it is to be regretted that a reference work of this type had to be produced with inferior printing materials.

KLAUS HOFMANN, Pittsburgh, Pa.

**The Chemical Senses.** By R. W. MONCRIEFF, B.Sc., A.R.I.C., F.T.I. Leonard Hall Limited, 17 Stratford Place, London, W. 1. 1944. vii+424 pp. Price \$3.50. Bound in dark green cloth.

Under this title the author includes not only Taste and Smell as we generally understand these words, but also what he terms "the common Chemical Sense," which responds to the action of irritating substances upon the mucous membranes and concerns not only the olfactory but other nerves (for example, the trigeminus) as well. This "chemical sense" he regards as a division of the sense of smell. G. H. Parker, in his "Smell, Taste, and Allied Senses in the Vertebrates," was the first to maintain that this reaction to chemical irritants was due to a sense which was separate and distinct from either Smell or Taste.

The Chemical Senses as thus defined are then reviewed in their physical, physiological, pathological, psychological, biological, chemical, biochemical, esthetic and artistic properties, associations and relationships, as they concern lower animals as well as man.

A list of the chapter headings will give the reader an idea of the extensive field which the author seeks to cover: (1) The Chemical Senses. Their Part in Life, (2) Structure of the Chemical Sense Organs and their Connections, (3) Sensation, (4) Olfaction, (5) Gustation, (6) The Common Chemical Sense, (7) Chemical Sensibility in Lower Animals, (8) Classification of Odours, (9) Chemical Constitution and Odour (10) Taste and Constitution, (11) Physical Properties of Odourous Materials, (12) Theories of Odour, (13) Perfumes and Essences, and (14) Flavour and Food.

The mass of data available in these various fields is so great that the author's chief problem must have been that of deciding what to include and what to reject, in order that the finished work should not exceed the compass of a single volume. The result is a very unusual birdseye view of a territory of such an extent as has hitherto required not one but many guide books to enable those interested to find their way therein. The chapter on Chemical Constitution and Odour, for example, is one of the most compact and comprehensive digests the reviewer has yet seen. In his discussion of sweetening agents, with Dulcin 200 times, Saccharin 675 times, and perilla *alpha*-anti-aldoxime 2000 times as sweet as cane sugar, the author will now have to add Verkade's 1-*n*-propoxy-2-amino-4-nitrobenzene claimed to be 4000 times as sweet as sugar and already manufactured on a large scale. He may also find it necessary to revise his Conclusions (pp. 271 *et seq.*) concerning the connection between chemical constitution and taste.

The text is followed by a Glossary of technical terms and a Bibliography. References to original articles are numerous throughout the text. The Author Index and Subject Index are excellent. Illustrations, graphs, structural formulas, and tables are numerous. Very few typographical errors were encountered.

The book is an exceptionally able résumé of the literature of the subject and as such is recommended to those interested.

MARSTON TAYLOR BOGERT, New York, N. Y.

**Electrophoresis by the Moving Boundary Method. A Theoretical and Experimental Study.** By HARRY SVENSSON; Arkiv För Kemi, Mineralogi Och Geologi, Band 22 A, No. 10 (1946), 156 pp. Almqvist & Wiksells Boktryckeri A.-B., Stockholm.

Dr. Harry Svensson has made many important contributions to electrophoretic technique. His monograph entitled "Electrophoresis by the Moving Boundary Method" will therefore be welcomed by all workers in this field.

The sections concerned with the design of analytical and preparative units are of general interest. Biochemists, especially, should find much use for the preparative apparatus which appears to be superior to any previously available. The main improvement is an arrangement that allows reversal of the electrode polarities with respect to the separation cell. This makes the process independent of electrode capacity and so permits continuous operation with great increase in separation efficiency.

Careful consideration is given in a long section to the optical problems involved in the Philpot-Svensson method of measuring refractive index gradients in the cell. Since these problems are for the most part common to the Longworth scanning method the analysis is pertinent to most electrophoretic equipment in current use, as well as to some other apparatus, such as diffusion cells, which may employ similar



optical systems. The calculations appear to be valid and useful. It would have been desirable from the mathematical point of view to have had some investigation of the convergence and remainder terms of the series employed for approximating the solutions of the differential equations. Perhaps, also, the deductions could have been somewhat more clearly organized to provide an elementary set of rules for adjustment of the optical system.

The account of electrophoretic migration theory appears to be substantially correct and adequate for its purpose. The moving boundaries formed by a protein-buffer system are under usual circumstances dominated by the slow moving massive protein ions. There are, however, appreciable influences of the smaller ions in any system and the theory apparently was developed to define conditions to minimize these complicating effects. Various qualitative deductions given will be particularly useful. Had the purpose been different the theory might have taken a more general and symmetrical form in which the system of moving boundaries would appear as an expression of the properties of all ions present in significant concentrations. The assumptions could also have been made slightly less restrictive. In any event it is important to realize that the existing theory becomes inaccurate when there is much variation among the relative values of the ionic mobilities in a system. This is likely to be the case with proteins or other weak electrolytes.

Electrophoretic methods in the future may be expected to be of value in a wide span of problems, ranging perhaps from data on some features of ionic interaction to purification of tissue enzymes. This monograph will improve understanding of the power and limitations of the moving boundary method.

V. P. DOLE, Boston, Mass.

**Colloids: Their Properties and Applications.** By A. G. WARD, M.A., formerly Scholar of Trinity College, Cambridge. Interscience Publishers, Inc., New York, N. Y., 1946. 133 pp. Price \$1.75. 28 fig. and 6 plates.

This little book consists of 3 parts. The first is headed "The nature of the colloidal state" but contains also a philosophical and historical introduction and chapters on the atomic structure of matter and on surfaces. Part II, the shortest of all, deals with "The colloidal systems," and Part III discusses "Colloids in industry and in living matter."

The inclusion of a chapter on "The atomic structure of matter" does not seem justified, the more so as this chapter is the longest in the book. Every author must decide for what kind of readers he is writing. If the readers of Mr. Ward's book are expected to be in need of such a review of the structure of matter, then they won't be able to understand many statements in the book which require much more knowledge than the above-mentioned chapter can impart. Compare, for instance, the following sentences: "The metal atoms which have lost electrons are called positive ions or cations, since, when solutions of salts of the metal are electrolyzed, these ions travel to the cathode" (p. 9). "A general rule for solubility is that like dissolves like, but the real criterion is that the loss of energy (if any) due to the change of neighbors on solution is more than compensated for by the increased disorder of an arrangement of two sorts of molecule" (p. 19). "Colloidal particles . . . have an average energy equal to that of the molecules. Owing to their large

size, the average velocity of the particles is very small" (p. 49). The first statement is elementary, but the second implies a relation between energy, free energy, entropy and disorder, while the third is based on the equation for the kinetic energy of particles. Neither that relation nor this equation could be found in the book by the reviewer.

The chapters dealing with colloids cover the usual range of subjects. The treatment is opposite to that recommended by many journal editors. These urge the authors to keep their experimental results (the permanent core) separated from the theoretical discussion (the fashionable garment). Mr. Ward weaves experiments and explanations into one fabric. I admit that the picture thus created might be easier to remember than are facts separated from their supposed meaning, but is this picture durable and true to life?

The explanations interwoven with experimental data are mainly of a "molecular" nature. It is stated, *e.g.*, on p. 29 that "after activation it (charcoal) will absorb large quantities of gases, particularly those with polar molecules . . . it adsorbs the poison gas, but not oxygen and nitrogen." The reader is not told that the adsorbability of a gas depends above all on the reduced temperature, *i.e.* on the ratio of the temperature of the experiment to the critical temperature of the gas. He is not told either that the liquid volume adsorbed from nearly saturated vapor is practically identical for polar ethanol and non-polar benzene. Several effects are attributed to the interaction between solute and solvent or between the particles and the surrounding liquid, although it is not stated how this interaction is measured and what independent phenomena are caused by it.

In Part III rubber, synthetic rubber, cellulose, proteins, *etc.*, are discussed. The knowledge of colloids which the reader gained in Part II is but little utilized here. Molecules, not colloidal particles, occupy the foreground. Sometimes the author describes colloidal materials (cellulose nitrate, cellulose acetate, *etc.*) from a purely industrial viewpoint. The chapter on "Colloids and living matter" is 3.5 pages long. Also in Part III "molecular" explanations are used. The author is impartial enough to say: ". . . the ideas given above . . . rest mainly on indirect rather than on direct evidence" (p. 94) and again: "the above views . . . are not yet accepted by all workers in this field" (p. 124). The reviewer applauds his critical attitude but doubts whether unsafe hypotheses should be awarded any space in a book which is so small for its task. The chapter on detergency is 5.2 p. long, and 1.5 p. of it is spent on "the above views."

The discussion of synthetic rubber, probably, would have been different if the book were compiled a little later. Although its date of publication is given as 1946, it appeared in Britain the year before, and I acquired a copy of it as early as July 1945. With the exception of the page on synthetic rubber this difference of a year has no importance: the author reviews the fundamentals of our science and they do not change so rapidly.

The make-up of the book is very pleasing, and the cuts and photographs are well reproduced.

J. J. BIKERMAN, New York, N. Y.

**Virus Diseases of Farm and Garden Crops.** By KENNETH M. SMITH, F.R.S. Plant Virus Research Station, School of Agriculture, Cambridge, England. Printed and Published by Littlebury and Company Ltd., The Worcester Press, Worcester, England, 1945. 111 pages, 16 plates, and 14 figures. Price 10 s. 6 d.

Farm and garden crops in all parts of the world are beset by virus diseases, the understanding and control of which will do much to improve production. Dr. Smith's new and well-written little volume dealing with this subject provides essential information in a convenient form. The text is prepared from the viewpoint of agriculture in the British Isles, yet the book will be valuable wherever truck crops are grown.

The book is bound in cloth. Most of its numerous illustrations are printed on a special paper, better suited to delineation of fine details than is the paper used for text pages.

The present volume is intended primarily for use by practical students, horticulturists, agricultural agents, and others to whom an introduction to the field is of importance. A more exhaustive treatment of particular viruses is found in the same author's earlier manual, *A Textbook of Plant Virus Diseases*, published in 1937.

Techniques of experimentation with viruses and their insect vectors are explained in the first two chapters. Then follow seven chapters describing the principal diseases caused by viruses in the potato, root crops (turnip and sugar beet), legume and pasture crops (pea, bean, broad bean, clovers), vegetables (cabbage, Chinese cabbage, carrot, celery, chicory, cucumber, horseradish, lettuce, onion, shallot, parsnip, radish, rhubarb, spinach, tomato, vegetable marrow and watercress), small fruits and hops (raspberry, strawberry, black currant, hop), ornamental, medicinal and miscellaneous plants. In an appendix, important insect vectors are portrayed by detailed drawings. An index, two and one half pages in length, provides ready access to information on each virus disease, insect vector, and host plant treated in the text.

Altogether this is an excellent treatise, small in size, skillfully written, well illustrated, and provided with a useful index.

FRANCIS O. HOLMES, Princeton, N. J.

**Preparation and Measurement of Isotopic Tracers**—A Symposium prepared for the Isotope Research Group. Lithoprinted by Edwards Brothers, Inc., Ann Arbor, Michigan, vii + 108 pp.

This small but highly compact compendium is a collection of papers on various technical and theoretical aspects of stable and radioactive isotopes. Its purpose, as stated by Dr. D. Wright Wilson in the preface to the book, is, "to acquaint biologists and chemists with the methods of preparation and methods of analysis of isotopes, especially carbon and hydrogen, which are of great potential value as tracers in studying organic chemical reactions and intermediary metabolism." There are eight papers in this volume, each contributed by an experienced investigator in the field under review: *The Separation of Stable Isotopes by Chemical Methods* by H. G. Thode and A. F. Reid; *The Mass Spectrometer and its Application to Isotope Abundance Measurements in Tracer Isotope Experiments* by A. O. Nier; *The Preparation of Gas Samples for Mass-Spectrographic Isotope Analysis* by D. Rittenberg; *The Preparation of Carbon Dioxide Samples for Isotope Analyses* by S. Weinhouse; *The Falling Drop Method for the Determination of Deuterium* by M. Cohn; *The Determination*

of Deuterium in the Gradient Tube by C. Anfinson; Production of the Radioactive Isotopes of Hydrogen, Carbon, and Sulfur by M. D. Kamen; and Detection and Measurement of Radioactive Tracers by A. F. Reid.

Each paper is well written and is designed to critically evaluate the methods and procedures described. The value of this treatment to beginners in the field is evident. Many of the papers include useful tables, figures and charts. This book is indispensable to every worker concerned with the use of isotopes, and is a source of profitable instruction admixed with some awe to those whose interest in the isotopes is only academic.

JESSE P. GREENSTEIN, Bethesda, Md.

**Anaerobiosis in Invertebrates.** By THEODOR VON BRAND, Professor of Biology in the Catholic University of America. No. 4 of the "Biodynamica Monographs" edited by B. J. Luyet. Biodynamica, Missouri, 1946. 328 pp. Price \$4.80.

That one as thoroughly versed in the study of invertebrate metabolism as von Brand has undertaken a review of the literature on invertebrate anoxybiosis in the form of a monograph, is to be deeply appreciated. Such a review is the more valuable because the literature on this subject is scattered in all types of periodicals: zoological, biochemical, physiological, ecological, etc.

The work consists of three parts; each containing several chapters. The first part is ecological and gives an extensive enumeration of the invertebrates which have been found in anaerobic surroundings, together with peculiarities on their mode of living in so far as this is of importance for the phenomenon of anoxybiosis. Extensive tables of the maximal time of survival under anaerobic conditions are given which enumerate all animals tested in this respect. In another chapter interesting figures are given on the composition of the gas phase in anaerobic habitats (soil, water basins, intestinal tract, tissues and secretions). This is important, especially for the gut, because so many contradictory results have been published. The author's conclusion is, that in fed animals the intestine of vertebrates is to be considered as a nearly anaerobic habitat (chiefly owing to the activity of bacteria). The oxygen contents of the soil are greatly dependent on the amount of water present. This is rapidly bereft of its small amount of oxygen by the action of bacteria and may thus give rise to a temporary anaerobic habitat. In this part some methods for the investigation of the amount of oxygen in air and water are also discussed as well as the means for experimentally producing anaerobic conditions.

The second part considers the phenomena of anoxybiosis from the standpoints of physiology and physiological chemistry. The best investigated processes of anoxybiosis (e.g., in *Ascaris*, *Fasciola*) are elaborately dealt with, together with important phenomena such as oxygen debt, recovery from anoxybiosis, primary and secondary aerobic processes, the sources of energy, the influence of oxygen tension on aerobic metabolic processes of facultative anoxybiotic animals, etc.

The third part deals with the general biological significance of anoxybiosis. A new classification of the organisms in this respect is proposed to replace that of von Brand and Harnisch (1933). Krogh's view, that there are, perhaps, no organisms which can exist without obtaining some slight trace of oxygen, is also discussed. Speculative, but theoretically highly interesting, is the discussion on the origin of anoxybiosis in

invertebrates, *viz.*, whether anoxybiosis is to be considered as the most primitive kind of metabolism or has been secondarily acquired. As a result of this discussion the author expresses his opinion that both mechanisms may have developed independently. Finally, the significance of anoxybiosis for the development of endoparasitism is treated.

The above summary of the contents may give an idea of the variety of subjects treated in this monograph and the many important problems which are related to the phenomenon of anoxybiosis.

As to the way in which the rich material is presented, I doubt whether the sequence which the author has chosen for the different chapters of Part II is the best didactical arrangement. In my opinion it would have been better to first treat what is known of the basic phenomena (Ch. III and IV of this part) and then Ch. I and II (transition to anaerobiosis and aerobic fermentations). Moreover, in some chapters the author has not escaped the danger of becoming rather too factual and has not avoided a certain repetition. Probably this is due to the fact that the book originated from some reviews which seem to have been previously published. However, this statement in no way tends to detract from the value of this book from the standpoints of both theory and experiment. It particularly stimulates the investigator to future work by stating clearly the very many unsolved problems which are still awaiting us in this field of research.

H. J. VONK, Utrecht

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